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Senior Scientist

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Education:

1978 - 1980 Postdoctoral Fellow in Immunology, Research Institute of Scripps Clinic,
Scripps Clinic and Research Foundation, La Jolla, California

1975 - 1978 Postdoctoral Fellow in Immunology, University of California at
San Francisco, San Francisco, California

1970 - 1975 Ph.D. in Microbiology, University of California at
Davis, California

1966 - 1970 B.A. in Biology/Microbiology, San Francisco State
University, San Francisco, California

Professional Positions:

Currently: Senior Scientist, Department of Immunology/Discovery Research, Genentech, Inc., South San Francisco, California

8/00-8/01 Acting Director, Department of Immunology, Genentech, Inc. South San Francisco, California

10/89 Senior Scientist in the Department of Immunology/Discovery Research, Genentech, Inc.
South San Francisco, California

3/89 - 10/89 Senior Scientist and Immunobiology Group Leader, Department of Pharmacological Sciences, Immunobiology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

9/87 - 3/89 Scientist, Department of Pharmacological Sciences, Immunopharmacology Section/Medical Research and Development, Genentech, Inc., S. San Francisco, California

1/82 - 9/87 Assistant Member (eq. Assistant Professor level), Department of Basic and Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

6/80 - 12/81 Scientific Associate in the Department of Clinical Research, Division of Clinical Immunology, Scripps Clinic and Research Foundation, La Jolla, California

7/78 - 6/80 Postdoctoral training in the laboratory of Dr. J. H. Vaughan, Chairman, Department of Clinical Research, Division of Clinical Immunology; Scripps Clinic and Research Foundation, La Jolla, California

2/75 - 6/78 Postdoctoral training in the laboratory of Dr. J. W. Goodman, Department of Microbiology and Immunology, School of Medicine, University of California, San Francisco, California

7/71 - 12/74 Research Assistant and Graduate Student, Department of Medical Microbiology, School of Medicine, University of California, Davis, California, under Dr. E. Benjamini

Awards:

Recipient: National Institutes of Health Postdoctoral Fellowship Award (1975).

Recipient: Special Research Award, (New Investigator Award), National Institute of Health (1980).

Recipient: P.I., Research Grant Award, National Institute of Health (1984).

Recipient: Research Career Development Award (R01), National Institutes of Health (1985).

Recipient: P.I., Multi-Purpose Arthritis Center Resarch Grant, NIH (1985)

Recipient: P.I., Resarch Grant Award, (R01 Renewal), National Institute of Health (1987).

Scientific Associations:

Sigma Xi, University of California, Davis, California Chapter

Member, The American Association of Immunologists

Committee Service and Professional Activities:

Member of the Immunological Sciences Study Section, National Institutes of Health Research Grant Review Committee, (1988-1992).

Advisory Committee, Scientific Review Committee for Veteran's Administration High Priority Program on Aging, 1983.

Ad Hoc member of Immunological Sciences Study Section, National Institutes of Health, 1988.

Ad Hoc Reviewer: Journal of Clinical Investigations, Journal of Immunology, Arthritis and Rheumatism, International Immunology, Molecular Cell Biology, and Gastroenterology

Biotechnology Experience

Established at Genentech in 1987-1989 within the Immunobiology Laboratory, in the Department of Pharmacological Sciences, group to study the immunogenicity of recombinant hGH (Protropin®) in hGH transgenic mice.

Served as Immunologist on the Biochemical Subteam for Protropin® Project team.

Served as Immunologist on the Met-less hGH and Dnase project teams, two FDA approved biological drugs: second generation hGH Nutropin® and Pulmozyme® (DNase).

Served immunologist in1989-1990 on the CD4-IgG project team carrying out in vitro immunopharmacological studies of the effects of CD4-IgG on the in vitro human immune responses to mitogens and antigens and on neutrophil responses in support of the filing of IND to FDA in 1990 for use of CD4-IgG in the prevention of HIV infection. Product was dropped.

In 1989-1991, initiated and carried research and development work on antibodies to CD11b and CD18 chains of the leukocyte β2 integrins. Provided preclinical scientific data to Anti-CD18 project team

supporting the advancement of humanized anti-CD18 antibody as anti-inflammatory in the acute setting. IND filed in 1996 and currently under clinical evaluation.

1993-1997, **Research Project Team leader** for small molecule $\alpha 4\beta 1$ integrin antagonist project. Leader for collaborative multidisciplinary team (N=11) composed of immunologists, molecular/cell biologists, protein engineers, pathologists, medicinal chemists, pharmacologists, pharmaceutical chemists, and clinical scientists targeting immune-mediated chronic inflammatory diseases. Responsible for research project plans and execution of strategy to identify lead molecules, assessment of biological activities, preclinical evaluation in experimental animals, and identification of potential clinical targets. Responsible for identification, hiring, and working with outside scientific consultants for project. Helped establish and responsible for maintaining current research collaboration with Roche-Nutley. Project transferred to Roche-Nutley.

1998-present, worked with Business Development to identify and create joint development opportunity with LeukoSite (currently Millennium) for monoclonal antibody against $\alpha 4\beta 7$ intergrin (LDP-02) for therapeutic treatment for inflammatory bowel disease (UC and Crohn's disease). Currently, working as scientific advisor to the core team for phase II clinical trials for LDP-02.

Currently, **Research Project Team Biology Leader** (1996-present) for small molecule antagonists for $\alpha 4\beta 7$ /MAdCAM-1 targeting the treatment of human inflammatory bowel diseases and diseases of the gastrointestinal tract. Responsible for leading collaborative team (N=12) from Departments of Immunology, Pathology, Analytical Technology, Antibody Technology, and Bio-Organic Chemistry to identify and evaluate lead drug candidates for the treatment of gastrointestinal inflammatory diseases.

Served for nearly fifteen years as **Ad Hoc reveiwer** on Genentech Internal Research Review Committee, Product Development Review Committee, and Pharmacological Sciences Review Committee.

Worked as **Scientific advisor** with staff of the **Business Development** Office on numerous occasions at Genentech, Inc. to evaluate the science of potential in-licensing of novel technologies and products.

2000-2001 Served as Research Discovery representative on Genentech Therapeutic Area Teams (Immunology/Endocrine, Pulmonary/Respiratory Disease Task Force)

Invited Symposium Lectures:

Session Chairperson and speaker, American Aging Association 12th Annual National Meeting, San Francisco, California, 1982.

Invited Lecturer, International Symposium, Mediators of Immune Regulation and Immunotherapy, University of Western Ontario, London, Ontario, Canada, 1985.

Invited Lecturer, workshop on Human IgG Subclasses, Rheumatoid Factors, and Complement. American Association of Clinical Chemistry, San Francisco, California, 1987.

Plenary Lecturer, First International Waaler Conference on Rheumatoid Factors, Bergen, Norway, 1987.

Invited Lecturer, Course in Immunorheumatology at the Universite aux Marseilles, Marseilles, France, 1988.

Plenary Lecturer, 5th Mediterranean Congress of Rheumatology, Istanbul, Turkey, 1988.

Invited Lecturer, Second Annual meeting of the Society of Chinese Bioscientist of America, University of California, Berkeley, California, 1988.

Lecturer at the inaugural meeting of the Immunology by the Bay sponsored by The Bay Area Bioscience Center. The β 2 Integrins in Acute Inflammation, July 14, 1992.

Lecturer, "Research and Development -- An Anatomy of a Biotechnology Company", University of California, Berkeley, Extension Course, given twice a year--March 9, 1995 to June 24, 1997.

Lecturer, "The Drug Development Process - Biologic Research - Genomics", University of California, Berkeley Extension, April 21, 1999, October, 1999, April 2000, October, 2000.

Lecturer, "The Drug Development Process - Future Trends/Impact of Pharmacogenomics", University of California Berkeley Extension, April 2001, October 2001, April 2002.

Invited Speaker, "Targeting of Lymphocyte Integrin α 4 β 7 Attenuates Inflammatory Bowel Diseases", in Symposium on "Nutrient effects on Gene Expression" at the Institute of Food Technology Symposium, June, 2002.

Patents:

Dennis A. Carson, Sherman Fong, Pojen P. Chen.

U.S. Patent Number 5,068,177: Anti-idiotype Antibodies induced by Synthetic Polypeptides, Nov. 26, 1991

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim and Steven R. Leong.

U.S. Patent Number 5,677,426: Anti-IL-8 Antibody Fragments, Oct. 14, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent Number 5,686,070: Methods for Treating Bacterial Pneumonia, Nov. 11, 1997

Claire M. Doerschuk, Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong. U.S. Patent 5,702,946: Anti-IL-8 Monoclonal Antibodies for the Treatment of Inflammatory Disorders, Dec. 30, 1997

Sherman Fong, Caroline A. Hebert, Kyung Jin Kim, Steven R. Leong.

U.S. Patent Number 5,707,622: Methods for Treating Ulcerative Colitis, Jan. 13, 1998

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,074,873: Nucleic acids encoding NL-3, June 13, 2000

Sherman Fong, Napoleone Ferrara, Audrey Goddard, Paul Godowski, Austin Gurney, Kenneth Hillan, and Mickey Williams. U.S. Patent Number 6,348,351 B1: The Receptor Tyrosine Kinase Ligand Homologues. February 19, 2002

Patent Applications:

Sherman Fong, Kenneth Hillan, Toni Klassen

U.S. Patent Application: "Diagnosis and Treatment of Hepatic Disorders"

Sherman Fong, Audrey Goddard, Austin Gurney, Daniel Tumas, William Wood

U.S. Patent Application: Compositions and Methods for the Treatment of Immune Related Diseases.

Sherman Fong, Mary Gerritsen, Audrey Goddard, Austin Gurney, Kenneth Hillan, Mickey Williams, William Wood . U.S. Patent Application: Promotion or Inhibition of Cardiovasculogenesis and Angiogenesis

Avi Ashkenazi, Sherman Fong, Audrey Goddard, Austin Gurney, Mary Napier, Daniel Tumas, William Wood. US Patent Application: Compounds, Compositions and Methods for the Treatment of Diseases Characterized by A33-Related Antigens

Chen, Filvaroff, Fong, Goddard, Godowski, Grimaldi, Gurney, Hillan, Tumas, Vandlen, Van Lookeren, Watanabe, Williams, Wood, Yansura

US Patent Application: IL-17 Homologous Polypeptides and Therapeutic Uses Thereof

Ashkenazi, Botstein, Desnoyers, Eaton, Ferrara, Filvaroff, Fong, Gao, Gerber, Gerritsen, Goddard, Godowski, Grimaldi, Gurney, Hillan, Kljavin, Mather, Pan, Paoni, Roy, Stewart, Tumas, Williams, Wood US Patent Application: Secreted And Transmembrane Polypeptides And Nucleic Acids Encoding The Same

Publications:

1. Scibienski R, Fong S, Benjamini E: Cross tolerance between serologically non-cross reacting forms of egg white lysozyme. *J Exp Med* 136:1308-1312, 1972.
2. Scibienski R, Harris M, Fong S, Benjamini E: Active and inactive states of immunological unresponsiveness. *J Immunol* 113:45-50, 1974.
3. Fong S: Studies on the relationship between the immune response and tumor growth. Ph D Thesis, 1975.
4. Benjamini E, Theilen G, Torten M, Fong S, Crow S, Henness AM: Tumor vaccines for immunotherapy of canine lymphosarcoma. *Ann NY Acad Sci* 277:305, 1976.
5. Benjamini E, Fong S, Erickson C, Leung CY, Rennick D, Scibienski RJ: Immunity to lymphoid tumors induced in syngeneic mice by immunization with mitomycin C treated cells. *J Immunol* 118:685-693, 1977.
6. Goodman JW, Fong S, Lewis GK, Kamin R, Nitecki DE, Der Balian G: T lymphocyte activation by immunogenic determinants. *Adv Exp Biol Med* 98:143, 1978.
7. Goodman JW, Fong S, Lewis GK, Kamin R, Nitecki DE, Der Balian G: Antigen structure and lymphocyte activation. *Immunol Rev* 39:36, 1978.
8. Fong S, Nitecki DE, Cook RM, Goodman JW: Spatial requirements of haptic and carrier determinants for T-dependent antibody responses. *J Exp Med* 148:817, 1978.
9. Fong S, Chen PP, Nitecki DE, Goodman JW: Macrophage-T cell interaction mediated by immunogenic and nonimmunogenic forms of a monofunctional antigen. *Mol Cell Biochem* 25:131, 1979.
10. Tsoukas CD, Carson DA, Fong S, Pasquali J-L, Vaughan JH: Cellular requirements for pokeweed mitogen induced autoantibody production in rheumatoid arthritis. *J Immunol* 125:1125-1129, 1980.
11. Pasquali J-L, Fong S, Tsoukas CD, Vaughan JH, Carson DA: Inheritance of IgM rheumatoid factor idiotypes. *J Clin Invest* 66:863-866, 1980.
12. Fong S, Pasquali J-L, Tsoukas CD, Vaughan JH, Carson DA: Age-related restriction of the light chain heterogeneity of anti-IgG antibodies induced by Epstein-Barr virus stimulation of human lymphocytes in vitro. *Clin Immunol Immunopathol* 18:344, 1981.
13. Fong S, Tsoukas CD, Frincke LA, Lawrence SK, Holbrook TL, Vaughan JH, Carson DA: Age-associated changes in Epstein-Barr virus induced human lymphocyte autoantibody responses. *J Immunol* 126:910-914, 1981.
14. Tsoukas CD, Fox RI, Slovin SF, Carson DA, Pellegrino M, Fong S, Pasquali J-L, Ferrone S, Kung P, Vaughan JH: T lymphocyte-mediated cytotoxicity against autologous EBV-genome-bearing B cells. *J Immunol* 126:1742-1746, 1981.
15. Fong S, Tsoukas CD, Pasquali J-L, Fox RI, Rose JE, Raiklen D, Carson DA, Vaughan JH: Fractionation of human lymphocyte subpopulations on immunoglobulin coated petri dishes. *J Immunol Methods* 44:171-182, 1981.
16. Pasquali J-L, Tsoukas CD, Fong S, Carson DA, Vaughan JH: Effect of Levamisole on pokeweed mitogen stimulation of immunoglobulin production in vitro. *Immunopharmacology* 3:289-298, 1981.

17. Pasquali J-L, Fong S, Tsoukas CD, Hench PK, Vaughan JH, Carson DA: Selective lymphocyte deficiency in seronegative rheumatoid arthritis. *Arthritis Rheum* 24:770-773, 1981.
18. Fong S, Fox RI, Rose JE, Liu J, Tsoukas CD, Carson DA, Vaughan JH: Solid-phase selection of human T lymphocyte subpopulations using monoclonal antibodies. *J Immunol Methods* 46:153-163, 1981.
19. Pasquali J-L, Fong S, Tsoukas CD, Slovin SF, Vaughan JH, Carson DA: Different populations of rheumatoid factor idiotypes induced by two polyclonal B cell activators, pokeweed mitogen and Epstein-Barr virus. *Clin Immunol Immunopathol* 21:184-189, 1981.
20. Carson DA, Pasquali J-L, Tsoukas CD, Fong S, Slovin SF, Lawrence SK, Slaughter L, Vaughan JH: Physiology and pathology of rheumatoid factors. *Springer Semin Immunopathol* 4:161-179, 1981.
21. Fox RI, Fong S, Sabharwal N, Carstens SA, Kung PC, Vaughan JH: Synovial fluid lymphocytes differ from peripheral blood lymphocytes in patients with rheumatoid arthritis. *J Immunol* 128:351-354, 1982.
22. Seybold M, Tsoukas CD, Lindstrom J, Fong S, Vaughan JH: Acetylcholine receptor antibody production during leukoplasmapheresis for Myasthenia Gravis. *Arch Neurol* 39:433-435, 1982.
23. Tsoukas CD, Fox RI, Carson DA, Fong S, Vaughan JH: Molecular interactions in human T-cell-mediated cytotoxicity to Epstein-Barr virus. I. Blocking of effector cell function by monoclonal antibody OKT3. *Cell Immunol* 69:113-121, 1982.
24. Sabharwal UK, Vaughan JH, Fong S, Bennett P, Carson DA, Curd JG: Activation of the classical pathway of complement by rheumatoid factors: Assessment by radioimmunoassay for C4. *Arthritis Rheum* 25:161-167, 1982.
25. Fox RI, Carstens SA, Fong S, Robinson CA, Howell F, Vaughan JH: Use of monoclonal antibodies to analyze peripheral blood and salivary gland lymphocyte subsets in Sjogren's Syndrome. *Arthritis Rheum* 25:419, 1982.
26. Fong S, Miller JJIII, Moore TL, Tsoukas CD, Vaughan JH, Carson DA: Frequencies of Epstein-Barr virus inducible IgM anti-IgG B lymphocytes in normal children and in children with Juvenile Rheumatoid Arthritis. *Arthritis Rheum* 25:959-965, 1982.
27. Goodman JW, Nitecki DE, Fong S, Kaymakcalan Z: Antigen bridging in the interaction of T helper cells and B cells. *Adv Exp Med Biol* 150:219-225, 1982.
28. Goodman JW, Nitecki DE, Fong S, Kaymakcalan Z: Antigen bridging in T cell-B cell interaction: Facts or fiction. In: Protein Conformation as Immunologic Signal. EMBO Workshop, Portonenere, Italy, 1982.
29. Tsoukas CD, Carson DA, Fong S, Slovin SF, Fox RI, Vaughan JH: Lysis of autologous Epstein-Barr virus infected B cells by cytotoxic T lymphocytes of rheumatoid arthritis patients. *Clin Immunol Immunopathol* 24:8-14, 1982.
30. Fong S, Vaughan JH, Tsoukas CD, Carson DA: Selective induction of autoantibody secretion in human bone marrow by Epstein-Barr virus. *J Immunol* 129:1941-1945, 1982.
31. Sabharwal UK, Fong S, Hoch S, Cook RD, Vaughan JH, Curd JG: Complement activation by antibodies to Sm in systemic lupus erythematosus. *Clin Exp Immunol* 51:317-324, 1983.

32. Tsoukas CD, Carson DA, Fong S, Vaughan JH: Molecular interactions in human T cell mediated cytotoxicity to EBV. II. Monoclonal antibody OKT3 inhibits a post-killer-target recognition/adhesion step. *J Immunol* 129:1421-1425, 1982.
33. Welch MJ, Fong S, Vaughan JH, Carson DA: Increased frequency of rheumatoid factor precursor B lymphocytes after immunization of normal adults with tetanus toxoid. *Clin Exp Immunol* 51:299-305, 1983.
34. Fong S, Vaughan JH, Carson DA: Two different rheumatoid-factor producing cell populations distinguished by the mouse erythrocyte receptor and responsiveness to polyclonal B cell activators. *J Immunol* 130:162-164, 1983.
35. Fox RI, Hueniken M, Fong S, Behar S, Royston I, Singhal SK, Thompson L: A novel cell surface antigen (T305) found in increased frequency on acute leukemia cells and in autoimmune disease states. *J Immunol* 131:762-767, 1983.
36. Fong S: Solid-phase panning for the fractionation of lymphoid cells. In: *Cell separation: methods and selected applications*. Pretlow TG, Pretlow TP (eds.) pp. 203-219. Academic Press, New York, 1983.
37. Carson DA, Fong S: A common idiotype on human rheumatoid factors identified by a hybridoma antibody. *Mol Immunol* 20:1081-1087, 1983.
38. Fong S, Gilbertson TA, Carson DA: The internal image of IgG in cross-reactive anti-idiotypic antibodies against human rheumatoid factors. *J Immunol* 131:719-724, 1983.
39. Fox RI, Adamson TC, Fong S, Young C, Howell FV: Characterization of the phenotype and function of lymphocytes infiltrating the salivary gland in patients with primary Sjogren syndrome. *Diagn Immunol* 1:233-239, 1983.
40. Fox RI, Adamson III TC, Fong S, Robinson CA, Morgan EL, Robb JA, Howell FV: Lymphocyte phenotype and function in pseudolymphoma associated with Sjogren's syndrome. *J Clin Invest* 72:52-62, 1983.
41. Fong S, Gilbertson TA, Chen PP, Vaughan JH, Carson DA: Modulation of human rheumatoid factor-specific lymphocyte responses with a cross-reactive anti-idiotype bearing the internal image of antigen. *J Immunol* 132:1183-1189, 1984.
42. Chen PP, Houghten RA, Fong S, Rhodes GH, Gilbertson TA, Vaughan JH, Lerner RA, Carson DA: Anti-hypervariable region antibody induced by a defined peptide. A new approach for studying the structural correlates of idiotypes. *Proc Natl Acad Sci USA* 81:1784-1788, 1984.
43. Fox RI, Fong S, Tsoukas CD, Vaughan JH: Characterization of recirculating lymphocytes in rheumatoid arthritis patients: Selective deficiency of natural killer cells in thoracic duct lymph. *J Immunol* 132:2883-2887, 1984.
44. Chen PP, Fong S, Normansell D, Houghten RA, Karras JG, Vaughan JH, Carson DA: Delineation of a cross-reactive idiotype on human autoantibodies with antibody against a synthetic peptide. *J Exp Med* 159:1502-1511, 1984.
45. Fong S, Carson DA, Vaughan JH: Rheumatoid factor. In: *Immunology of Rheumatic Diseases*. Gupta S, Talal N (eds.): Chapter 6. pp. 167-196. Plenum Publishing Corp., New York, 1985.

46. Fong S: Immunochemistry. In: Immunology as applied to Otolaryngology. Ryan AF, Poliquis JF, Harris A (eds.): pp. 23-53. College Hill Press, San Diego, 1985.
47. Fong S, Chen PP, Vaughan JH, Carson DA: Origin and age-associated changes in the expression of a physiologic autoantibody. *Gerontology* 31:236-250, 1985.
48. Chen PP, Fong S, Houghten RA, Carson DA: Characterization of an epitope: An anti-idiotype which reacts with both the idiotype of rheumatoid factors (RF) and the antigen recognized by RFs. *J Exp Med* 161:323, 1985.
49. Chen PP, Goni F, Fong S, Jirik F, Vaughan JH, Frangione B, Carson DA: The majority of human monoclonal IgM rheumatoid factors express a primary structure-dependent cross-reactive idiotype. *J Immunol* 134:3281-3285, 1985.
50. Lotz M, Tsoukas CD, Fong S, Carson DA, Vaughan JH: Regulation of Epstein-Barr virus infections by recombinant interferon. Selected sensitivity to interferon-gamma. *Eur J Immunol* 15:520-525, 1985.
51. Chen PP, Kabat EA, Wu TT, Fong S, Carson DA: Possible involvement of human D-minigenes in the first complementarity-determining region of kappa light chains. *Proc Natl Acad Sci USA* 82:2125-2127, 1985.
52. Goldfien RD, Chen PP, Fong S, Carson DA: Synthetic peptides corresponding to third hypervariable region of human monoclonal IgM rheumatoid factor heavy chains define an immunodominant idiotype. *J Exp Med* 162:756-761, 1985.
53. Fong S, Chen PP, Gilbertson TA, Fox RI, Vaughan JH, Carson DA: Structural similarities in the kappa light chains of human rheumatoid factor paraproteins and serum immunoglobulins bearing a cross-reactive idiotype. *J Immunol* 135:1955-1960, 1985.
54. Chen PP, Goni F, Houghten RA, Fong S, Goldfien RD, Vaughan JH, Frangione B, Carson DA: Characterization of human rheumatoid factors with seven antiidiotypes induced by synthetic hypervariable-region peptides. *J Exp Med* 162:487-500, 1985.
55. Fong S, Gilbertson TA, Hueniken RJ, Singhal SK, Vaughan JH, Carson DA: IgM rheumatoid factor autoantibody and immunoglobulin producing precursor cells in the bone marrow of humans. *Cell Immunol* 95:157-172, 1985.
56. Fong S, Chen PP, Goldfien RD, Jirik F, Silverman G, Carson DA: Recurrent idiotypes of human anti-IgG autoantibodies: their potential use for immunotherapy. In: *Mediators of Immune Regulation and Immunotherapy*. Singhal SK, Delovitch TL (eds.): pp. 232-243. Elsevier Science Publishing Co., New York, 1986.
57. Fox RI, Chen PP, Carson DA, Fong S: Expression of a cross reactive idiotype on rheumatoid factor in patients with Sjogren's syndrome. *J Immunol* 136:477-483, 1986.
58. Jirik FR, Sorge J, Fong S, Heitzmann JG, Curd JG, Chen PP, Goldfien R, Carson DA: Cloning and sequence determination of a human rheumatoid factor light-chain gene. *Proc Natl Acad Sci USA*, 83:2195-2199, 1986.
59. Lotz M, Tsoukas CD, Fong S, Dinarello CA, Carson DA, Vaughan JH: Release of lymphokines following Epstein-Barr virus infection in vitro. I. The sources and kinetics of production of interferons and interleukins in normal humans. *J Immunol*, 136:3636-3642, 1986.

60. Lotz M, Tsoukas CD, Fong S, Dinarello CA, Carson DA, Vaughan JH: Release of lymphokines following infection with Epstein-Barr virus in vitro. II. A monocyte dependent inhibitor of interleukin-1 downregulates the production of interleukin-2 and gamma interferon in rheumatoid arthritis. *J Immunol*, 136:3643-3648, 1986.
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62. Fong S, Gilbertson TA, Chen PP, Karras JG, Vaughan JH, Carson DA: The common occurrence of internal image type anti-idiotypic antibodies in rabbits immunized with monoclonal and polyclonal human IgM rheumatoid factors. *Clin Exp Immunol*, 64:570-580, 1986.
63. Fox RI, Carson DA, Chen P, Fong S: Characterization of a cross reactive idotype in Sjogren's syndrome. *Scand J Rheum* S61:83-88, 1986.
64. Silverman GJ, Carson DA, Solomon A, Fong S: Human kappa light chain subgroup analysis with synthetic peptide-induced antisera. *J Immunol Methods* 95:249-257, 1987.
65. Goldfien R, Chen P, Kipps TJ, Starkebaum G, Heitzmann JG, Radoux V, Fong S, Carson DA: Genetic analysis of human B cell hybridomas expressing a rheumatoid factor-associated cross-reactive idotype. *J Immunol* 138:940- 944, 1987.
66. Carson DA, Chen PP, Fox RI, Kipps TJ, Jirik F, Goldfien RD, Silverman G, Radoux V, Fong S: Rheumatoid factors and immune networks. *Annu Rev Immunol* 5:109-126, 1987.
67. Kipps TJ, Fong S, Tomhave E, Chen PP, Goldfien RD, Carson DA: High frequency expression of a conserved kappa variable region gene in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 84:2916-2920, 1987
68. Fong S, Chen PP, Fox RI, Goldfien RD, Silverman GJ, Radoux V, Jirik F, Vaughan JH, Carson DA: Rheumatoid factors in human autoimmune disease: Their origin, development and function. *Path Immunopath Res* 5:407-449, 1987.
69. Goldfien RD, Fong S, Chen P, Carson DA: Structure and function of rheumatoid factor: Implications for its role in the pathogenesis of mixed cryoglobulinemia. In: *Antiglobulins, cryoglobulins and glomerulonephritis*, Proceedings of 2nd International Milano Meeting of Nephrology. Ponticelli C, Minetti L, D'Amico G (eds.) Martinus Nijhoff, Dordrecht pp. 17-27, 1986.
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71. Silverman GJ, Fong S, Chen PP, Carson DA: Clinical update: Cross reactive idotype and the genetic origin of rheumatoid factors. *J Clin Lab Anal*, 1:129-135, 1987.
72. Chen PP, Fong S, Carson DA: Molecular basis of reactivity of epitopes. In: *Elicitation and use of anti-idiotypic antibodies and their biological applications*. Bona C (ed.), CRC Press, Boca Raton, Fla, in press.
73. Fong S, Chen PP, Carson DA, Fox, RI: Rheumatoid factor in Sjogren's syndrome. In: *Sjogren's Syndrome: Clinical and Immunological Aspects*. Talal N, Moutsopoulos HM, Kassan SS (eds.), Springer-Verlag, New York, pp. 203-217, 1987.

74. Chen PP, Fong S, Carson DA: The use of defined peptides in characterizing idiotypes. *Int Rev Immunol* 2:419-432, 1987.
75. Chen PP, Fong S, Goni F, Houghten RA, Frangione B, Liu F, Carson DA: Analyses of human rheumatoid factors with anti-idiotypes induced by synthetic peptides. *Monogr Allergy* 22:12-23, 1987.
76. Carson DA, Chen PP, Radoux V, Jirik F, Goldfien RD, Silverman GJ, Fong S: Molecular basis for the cross-reactive idiotypes on human anti-IgG autoantibodies (rheumatoid factors) In: Autoimmunity and Autoimmune Disease. Ciba Foundation Symposium 129 pp. 123-130, 1987.
77. Radoux V, Fong S, Chen PP, Carson DA: Rheumatoid factors: current concepts. In: Advances in Inflammation Research. Lewis, A. (Ed), Raven Press, New York, pp.295-304, 1987.
78. Kipps TJ, Fong S, Chen PP, Miller WE, Piro LD, Carson DA: Immunoglobulin V gene utilization in CLL. In: Chronic lymphocytic leukemia: recent progress and future direction, Alan R Liss, Inc., New York, pp. 115-126, 1987.
79. Fong S, Chen PP, Fox RI, Goldfien RD, Radoux V, Silverman GJ, Crowley JJ, Roudier J, Carson DA: The diversity and idiotypic pattern of human rheumatoid factors in disease. *Concepts in Immunopath* 5: 168-191, 1988.
80. Fox RI, Fong S, Chen PP, Kipps TJ: Autoantibody production in Sjogren's syndrome: a hypothesis regarding defects in somatic diversification of germline encoded genes. *In Vivo* 2: 47-56, 1988.
81. Chen PP, Fong S, Goni F, Silverman GJ, Fox RI, Liu M-K, Frangione B, Carson DA: Cross-reacting idiotypes on cryoprecipitating rheumatoid factor. *Springer Seminar Series in Immunopath* 10: 35-55, 1988.
82. Chen PP, Fong S, Carson DA: Rheumatoid factor. *Rheumatic Diseases of North America* 13:545-568, 1987.
83. Carson DA, Chen PP, Kipps TJ, Radoux V, Jirik FR, Goldfien RD, Fox RI, Silverman GJ, Fong S. Idiotype and genetic studies of human rheumatoid factors. *Arthritis Rheum* 30: 1321-1325, 1987.
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INTERLEUKIN-12 (IL-12)-DRIVEN ALLOIMMUNE RESPONSES IN VITRO AND IN VIVO

REQUIREMENT FOR $\beta 1$ SUBUNIT OF THE IL-12 RECEPTOR¹

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Background. Interleukin-12 (IL-12) mediates its biologic activities via binding high-affinity receptors on T and natural killer cells. Although emphasis has been placed on the requirement for IL-12R $\beta 2$ in IL-12 bioactivity, the role of IL-12R $\beta 1$ is less well defined. The current study evaluated the effects of exogenous IL-12 on alloantigen-specific immune responses and determined the requirement for IL-12R $\beta 1$ in IL-12-mediated alloimmunity.

Methods. The mouse heterotopic cardiac transplant model was employed to evaluate the effects of IL-12 on alloantigen-specific immune responses *in vivo*. In addition, IFN- γ production in mixed lymphocyte cultures (MLC) supplemented with IL-12 was measured to assess the effects of IL-12 on Th1 function *in vitro*. Mice deficient in IL-12R $\beta 1$ (IL-12R $\beta 1^{-/-}$) were used to determine the requirement for this receptor component in IL-12-driven alloimmune responses.

Results. Addition of IL-12 to MLC consisting of wild-type splenocytes enhanced alloantigen-specific proliferative responses and Th1 development. In contrast, IL-12 did not alter these *in vitro* immune parameters in IL-12R $\beta 1^{-/-}$ MLC. Treatment of wild-type cardiac allograft recipients with IL-12 resulted in high concentrations of serum interferon- γ (IFN- γ) and a 10-fold increase in IFN- γ production by recipient splenocytes after restimulation *in vitro*. However, this fulminate Th1 response did not accelerate allograft rejection. Importantly, IL-12 had no effect on serum IFN- γ or *in vivo* priming of Th1 in IL-12R $\beta 1^{-/-}$ recipients. Finally, administration of IL-12 to WT allograft recipients resulted in a bimodal alloantibody response: antibody production was suppressed at high doses of IL-12, and enhanced at lower doses.

Conclusions. IL-12 markedly enhances alloantigen-specific immune function; however, these exaggerated Th1-driven responses do not culminate in accelerated allograft rejection. Further, these data indicate that IL-12R $\beta 1$ is essential for the enhancement of both *in vitro* and *in vivo* alloimmune responses by exogenous IL-12.

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vitro and in vivo alloimmune responses by exogenous IL-12.

It is well established that interleukin-12 (IL-12*) is a critical cytokine involved in the regulation of Th1- and Th2-mediated immune responses in several experimental models (reviewed in 1 and 2). IL-12 has direct stimulatory and inhibitory effects on Th1 and Th2, respectively (3-6). Further, this cytokine promotes Th1 and inhibits Th2 development indirectly by inducing interferon- γ (IFN- γ) production by activated T cells and natural killer cells (7-12). Th1 have been accepted as key regulators of allograft rejection, in that this cell type promotes both delayed-type hypersensitivity and cytotoxic T lymphocyte responses, which are believed to be the principle terminal effector mechanisms of acute allograft rejection (13, 14). An understanding of the role of IL-12 in graft rejection is just emerging. For example, IL-12 clearly augments alloreactive Th1 development *in vitro* (15). However, the presence of IL-12 is not mandatory for the development of acute cardiac allograft rejection (15, 16). Hence, an important question is whether enhanced Th1 function alters the rejection response. Given the IL-12/Th1 dogma, one would predict that IL-12 would augment alloreactive Th1 function, resulting in accelerated allograft rejection. The present study therefore was designed to test the hypothesis that IL-12-driven Th1 responses would exacerbate cardiac allograft rejection.

IL-12 mediates its biologic effects by interacting with a high-affinity receptor, which consists of at least two cloned components, IL-12R $\beta 1$ and IL-12R $\beta 2$ (17-19). IL-12R $\beta 1$ interacts with the p40 subunit of IL-12, whereas the p35 subunit of IL-12 is believed to bind to IL-12R $\beta 2$ (19, 20). Emphasis has been placed on the necessity for IL-12R $\beta 2$ in IL-12 signaling (21, 22). However, by utilizing IL-12R $\beta 1$ knockout mice (IL-12R $\beta 1^{-/-}$), Wu et al. (23) recently reported that the $\beta 1$ subunit of IL-12R is essential for IL-12-driven proliferation and IFN- γ production by mitogen-activated blasts, natural killer cell lytic activity, and IFN- γ production in response to endotoxin. We have reported that the p40 subunit of IL-12 stimulates alloreactive CD8+ Th1 development both *in vitro* (24) and *in vivo* (15). These observations suggest that

* Abbreviations: Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; GVHD, graft-versus-host disease; H&E, hematoxylin and eosin; IFN- γ , interferon- γ ; IL, interleukin IL-12R $\beta 1^{-/-}$, mice deficient in $\beta 1$ subunit of IL-12 receptor; mAb, monoclonal antibody; MLC, mixed lymphocyte culture; WT, wild-type.

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IL-12R β 1 may be needed for alloreactive Th1 development, and that signaling through IL-12R β 1 may be sufficient to mediate IL-12's biologic activity on CD8+ T cells. Hence, the present study employed IL-12R β 1 $^{-/-}$ mice to determine whether β 1 subunit of IL-12R is required for IL-12-induced alloantigen-specific immune responses. To our knowledge, this study is the first to investigate the effects of IL-12 treatment on alloreactive Th1 development *in vivo* and to establish a mandatory role for IL-12R β 1 in IL-12-driven alloimmune responses.

MATERIALS AND METHODS

Mice. Wild-type (WT) C57BL/6 and BALB/c mice between 6 and 12 weeks of age were obtained from Charles River Laboratories (Raleigh, NC). Generation of C57BL/6 IL-12R β 1 $^{-/-}$ mice has been described previously (23). These mice were generated on the 129/Sv background and back-crossed to C57BL/6 mice for five generations, then intercrossed to generate homozygotes.

Medium. The culture medium used in these studies was Dulbecco's minimum essential medium supplemented with 1.6 mM L-glutamine, 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μ M folic acid, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, 2% fetal calf serum (all obtained from Life Technologies, Grand Island, NY), and 5 \times 10 $^{-5}$ M 2-mercaptoethanol (Sigma Chemical, St Louis, MO).

Mitogen-driven cytokine production. To investigate the requirement for the β 1 subunit of IL-12R in mitogen-stimulated IL-10 and IFN- γ production, splenocytes (2 \times 10 6 cells/ml) isolated from naive WT or IL-12R β 1 $^{-/-}$ C57BL/6 mice were incubated for 72 hr with 1 μ g/ml concanavalin A (Con A) (Sigma Chemical). Cultures were supplemented with 1 ng/ml murine recombinant IL-12 (rIL-12) (kindly provided by Dr. Maurice Gately, Hoffmann-La Roche Inc.) to assess the effect of exogenous IL-12 on Con A-stimulated cytokine production by splenocytes of WT and IL-12R β 1-deficient mice. Resulting supernatants were harvested at 72 hr, and the concentrations of IL-10 and IFN- γ measured by enzyme-linked immunosorbent assay (ELISA).

In vitro alloimmune responses. To assess alloantigen-specific Th1 development, splenocytes (1 \times 10 6 cells/ml) isolated from naive WT or IL-12R β 1 $^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated (5000 rads) BALB/c splenocytes (1 \times 10 6 cells/ml). Where indicated, 1 ng/ml of murine rIL-12 was added to primary mixed lymphocyte cultures (MLC) to assess the effect of exogenous IL-12 on alloantigen-driven Th1 function and to evaluate whether Th1 from IL-12R β 1-deficient mice were responsive to IL-12 stimulation. The concentration of rIL-12 was selected from dose-response experiments in which the amount of rIL-12 needed for maximal enhancement of alloantigen-specific proliferation was 5–10 ng/ml (data not shown). Resulting cell populations were harvested, washed three times, and restimulated (at 1 \times 10 6 cells/ml) with irradiated BALB/c stimulator cells (1 \times 10 6 cells/ml). MLC supernatants were collected after 24 hr (IL-4 and IL-10) or 72 hr (IFN- γ), and cytokine concentrations measured by ELISA.

In addition, splenocyte proliferative response to alloantigens was determined in cultures either left unmodified or supplemented with 1 ng/ml murine rIL-12. WT or IL-12R β 1 $^{-/-}$ C57BL/6 splenocytes (1 \times 10 6 cells/ml) were stimulated for 5 days with irradiated BALB/c splenocytes (1 \times 10 6 cells/ml) in 96-well U-bottom plates (Becton Dickinson, Lincoln Park, NJ) in a final volume of 200 μ l (done in quadruplicate). Cultures were pulsed with 0.5 μ Ci/well [methyl- 3 H]thymidine (ICN, Costa Mesa, CA) for the final 8 hr of the incubation period. [methyl- 3 H]Thymidine incorporation was assessed on a Wallac 1205 Betaplate scintillation counter (Wallac, Turku, Finland).

Heterotopic cardiac transplantation. Intact BALB/c (H2 d) hearts were anastomosed to the great vessels in the abdomens of WT or

IL-12R β 1 $^{-/-}$ C57BL/6 (H2 b) mice as described by Corry et al. (25). In this model, the transplanted heart is perfused with the recipient's blood and resumes contractions until acutely rejected, which occurs in unmodified WT recipients of this strain combination in approximately 8–9 days (15, 24). Graft function was evaluated by daily abdominal palpation. Myocyte damage and intensity of graft-infiltrating cells were assessed by routine hematoxylin and eosin (H&E) staining of paraffin-embedded sections of transplanted allografts.

Experimental groups. Cardiac allograft recipients were divided into four groups: (1) recipients injected intraperitoneally with 1 mg of anti-CD8 monoclonal antibody (mAb) (hybridoma 2.43, purified by Montana ImmunoTech Inc., Bozeman, MT) on days –2 and –1 before transplantation, (2) animals given daily intraperitoneal injections of murine rIL-12 (0.1 or 1.0 μ g) on days 1–6 after transplantation, (3) recipients injected with a combination of 2.43 anti-CD8 mAb plus rIL-12, and (4) unmodified (no treatment) mice, which served as controls. Depletion of CD8+ cells (<2%) was verified by flow cytometry using anti-CD8:fluorescein isothiocyanate antibody (PharMingen).

In vivo alloimmune responses. To monitor in vivo Th1 development, splenocytes (1 \times 10 6 cells/ml) obtained from allograft recipients were restimulated with irradiated BALB/c stimulator cells (1 \times 10 6 cells/ml), and the concentration of IFN- γ was measured by ELISA. As an additional measure of the in vivo activity of IL-12 on IFN- γ production, sera IFN- γ concentrations in WT and IL-12R β 1 $^{-/-}$ cardiac allograft recipients were measured by ELISA. Further, to assess the effect of IL-12 treatment on B cell function, sera alloantibody (IgM, IgG1, and IgG2a) levels were determined (see below).

Cytokine ELISA. Experimental samples (100 μ l) were added in triplicate to plates coated with 5 μ g/ml rat anti-mouse IFN- γ , IL-4, or IL-10 capture antibodies (PharMingen). Standards were employed by preparing 2-fold dilutions of murine recombinant IFN- γ , IL-4, and IL-10 (PharMingen), with a starting concentration of 25, 2.5, and 10 ng/ml, respectively. After a 1-hr incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS. One hundred microliters of rat anti-mouse secondary biotinylated antibodies (1 μ g/ml) (PharMingen) was then added, and plates were incubated at room temperature for 45 min. Plates were then washed three times with 0.05% Tween 20 in PBS, and 100 ml of avidin-peroxidase (Sigma Chemicals) was added. After a 30-min incubation at room temperature, plates were washed three times with 0.05% Tween 20 in PBS, and 100 ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma Chemical) was then added to each well. After 20 min, absorbance was determined at 405 nm by an EL 800 microplate reader (Bio-Tek Instruments, Winooski, VT). Sample cytokine concentrations were calculated from a standard curve. The sensitivity of this assay is approximately 300 pg/ml for IFN- γ , 100 pg/ml for IL-4, and 150 pg/ml for IL-10.

Sera alloantibody determination. P815 cells (H2 d) were stained for flow cytometric analysis using dilutions of sera (1:50) obtained from cardiac allograft recipients as the primary antibody, followed by fluorescein isothiocyanate-conjugated isotype-specific anti-mouse IgM, IgG1, and IgG2a secondary antibodies (The Binding Site, San Diego, CA). Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACScan.

Statistics. Statistical analyses in this study were done using a Student's *t* test performed by the program StatView 4.1.

RESULTS

Requirement for IL-12R β 1 in T Cell Responses *in Vitro*

Enhancement of mitogen-driven IFN- γ and IL-10 production by IL-12 requires IL-12R β 1. IL-12 stimulates concomitant production of IL-10 and IFN- γ by activated T cells (15, 26, 27). To determine whether β 1 subunit of IL-12R is required for production of these cytokines, C57BL/6 splenocytes isolated WT or IL-12R β 1-deficient mice were stimu-

lated with Con A for 72 hr, and supernatant cytokine concentrations were determined by ELISA. Production of the Th1 cytokine IFN- γ by Con A-stimulated splenocytes isolated from IL-12R $\beta 1^{-/-}$ mice was readily detectable (Fig. 1A), although concentrations were lower than that seen in WT controls (IL-12R $\beta 1^{-/-}$ = 1.03 ng/ml vs. WT = 5.24 ng/ml). Addition of exogenous rIL-12 significantly enhanced IFN- γ production by mitogen-stimulated splenocytes obtained from WT mice (15.79 ng/ml). In contrast, IFN- γ production by splenocytes from IL-12R $\beta 1^{-/-}$ mice was not altered after the addition of rIL-12 (1.54 ng/ml).

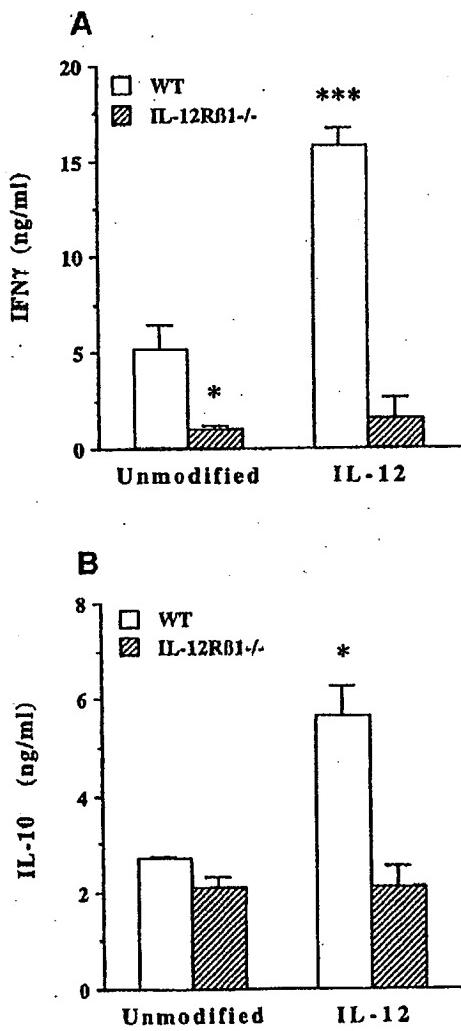


FIGURE 1. Mitogen-driven cytokine production by splenocytes isolated from IL-12R $\beta 1^{-/-}$ mice. Splenocytes (2×10^6 cells/ml) obtained from WT or IL-12R $\beta 1^{-/-}$ C57BL/6 mice were stimulated in vitro with 1 μ g/ml Con A. Cultures were either left untreated or supplemented with murine rIL-12 (1 ng/ml). Supernatants were collected after 72 hr, and the concentrations of IFN- γ (A) and IL-10 (B) were determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples \pm SD. Data are representative of three separate experiments. In panel A, * $P < 0.05$ (WT unmodified vs. IL-12R $\beta 1^{-/-}$ unmodified); *** $P < 0.005$ (WT unmodified vs. WT IL-12-treated). In panel B, * $P < 0.05$ (WT unmodified vs. WT IL-12-treated).

The requirement for IL-12R $\beta 1$ in IL-12-driven IL-10 production also was assessed. Splenocytes isolated from IL-12R $\beta 1$ -deficient mice produced similar levels of IL-10 upon Con A stimulation when compared to WT cells (Fig. 1B). rIL-12 enhanced Con A-stimulated IL-10 production by WT splenocytes (2.70 ng/ml vs. 5.65 ng/ml). However, the $\beta 1$ subunit of IL-12R was required for this response, as IL-12 did not affect IL-10 secretion by mitogen-stimulated IL-12R $\beta 1^{-/-}$ splenocytes.

In vitro alloreactive T helper cell development. To evaluate the requirement for IL-12R $\beta 1$ in IL-12-driven alloantigen-specific T cell development, naive splenocytes obtained from WT or IL-12R $\beta 1^{-/-}$ mice were incubated for 5 days with irradiated BALB/c splenocytes in primary MLC, which were either left unmodified or supplemented with rIL-12. Resulting cell populations were restimulated with irradiated BALB/c splenocytes in the absence of rIL-12, and in vitro IFN- γ , IL-4, and IL-10 production determined by ELISA (Table 1). Primed WT splenocytes secreted high levels of IFN- γ upon restimulation with alloantigens. Splenocytes obtained from IL-12R $\beta 1^{-/-}$ mice secreted IFN- γ upon restimulation with alloantigens, albeit to a lesser degree than WT cells (WT = 21.32 ng/ml vs. IL-12R $\beta 1^{-/-}$ = 5.72 ng/ml). The decrease in alloantigen-stimulated IFN- γ production in IL-12R $\beta 1$ -deficient mice was not associated with a decrease in the cells' ability to proliferate in response to alloantigens (Fig. 2), in that [³H]thymidine incorporation by alloantigen-stimulated IL-12R $\beta 1^{-/-}$ splenocytes was similar to that seen by WT cells (IL-12R $\beta 1^{-/-}$ = 13,385 cpm vs. WT = 11,441 cpm). In both groups, IL-4, IL-10 (Table 1), and IL-5 (data not shown) were not detected in cultures that were not supplemented with exogenous rIL-12.

As shown in Table 1, exogenous rIL-12 markedly enhanced IFN- γ production by WT splenocytes in vitro (21.32 ng/ml vs. 215.13 ng/ml), but failed to augment IFN- γ secretion by cells obtained from IL-12R $\beta 1^{-/-}$ mice (5.72 ng/ml vs. 6.80 ng/ml). Likewise, rIL-12 significantly enhanced WT splenocyte proliferation in the MLC (Fig. 2), but did not alter the proliferative ability of splenocytes isolated from IL-12R $\beta 1$ -deficient mice. Finally, the addition of exogenous rIL-12 to cultures

TABLE 1. IL-12 does not enhance alloantigen-specific Th1 development in IL-12R $\beta 1^{-/-}$ mice in vitro^a

Treatment	IFN- γ (ng/ml)	IL-4 (ng/ml)	IL-10 (ng/ml)
Unmodified			
WT	21.32 \pm 0.88	ND	ND
IL-12R $\beta 1^{-/-}$	5.72 \pm 0.24**	ND	ND
IL-12 (1 ng/ml)			
WT	215.13 \pm 13.52***	ND	1.54 \pm 0.21
IL-12R $\beta 1^{-/-}$	6.80 \pm 0.21	ND	ND

^a Splenocytes (1×10^6 cells/ml) obtained from WT or IL-12R $\beta 1^{-/-}$ C57BL/6 mice were incubated for 5 days with irradiated allogeneic splenocytes (1×10^6 cells/ml) in unmodified MLC or MLC supplemented with murine rIL-12 (1 ng/ml). Resulting cell populations were harvested and restimulated with alloantigens for cytokine determination. Supernatants concentrations of IFN- γ (72 hr), IL-4 (24 hr), and IL-10 (24 hr) were measured by ELISA. Results are expressed as the cytokine concentration in triplicate samples (mean \pm SD). Data are representative of four separate experiments. ND indicates not detectable. ** $P < 0.01$, WT unmodified vs. IL-12R $\beta 1^{-/-}$ unmodified; *** $P < 0.005$, WT unmodified vs. WT IL-12-treated.

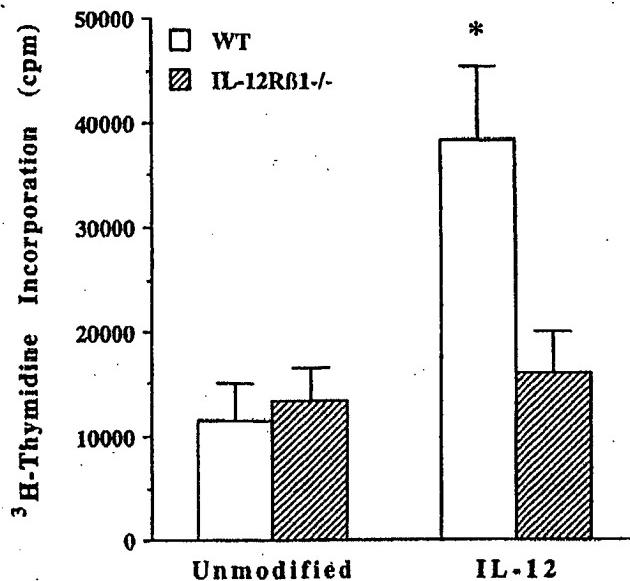


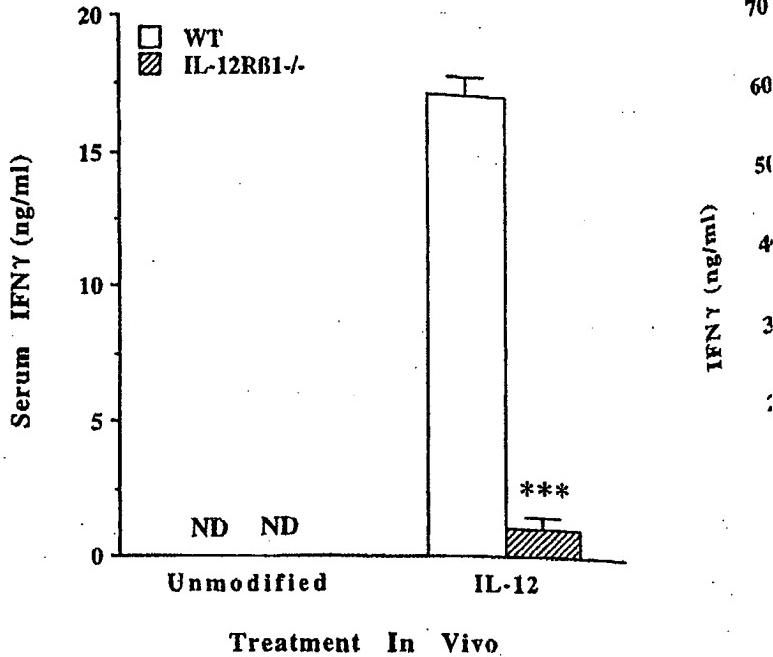
FIGURE 2. The $\beta 1$ subunit of IL-12 receptor is required for IL-12-induced stimulation of alloantigen-specific splenocyte proliferation. C57BL/6 splenocytes (1×10^6 cells/ml) were stimulated with irradiated allogeneic BALB/c splenocytes (1×10^6 cells/ml) in 96-well microtiter plates for 5 days. Cultures were pulsed with $0.5 \mu\text{Ci}/\text{well}$ [methyl^3H]thymidine for the final 8 hr of the incubation period, and thymidine incorporation was determined by liquid scintillation spectrophotometry. Results are expressed as the mean cpm in quadruplicate samples \pm SD. Data are representative of three separate experiments. *, $P < 0.05$ (WT unmodified vs. WT IL-12-treated).

stimulated the secretion of IL-10 by alloantigen-stimulated WT splenocytes, but not IL-12R $\beta 1^{-/-}$ cells (Table 1). Collectively, these data indicate that the $\beta 1$ subunit of IL-12R is required for the enhancement of several in vitro alloimmune responses by exogenous rIL-12, including increased alloantigen-stimulated T cell proliferation, and IFN- γ and IL-10 production.

Effects of Exogenous IL-12 on Alloimmune Responses in Vivo

Enhancement of serum IFN- γ by IL-12 treatment. To monitor the in situ effects of IL-12 treatment on IFN- γ production in cardiac allograft recipients, serum IFN- γ concentrations were measured on day 7 after transplantation (Fig. 3). In both WT and IL-12R $\beta 1^{-/-}$ allograft recipients, serum IFN- γ was undetectable by ELISA on day 7 after transplantation. Treatment of WT recipients with rIL-12 markedly increased serum IFN- γ in three independent experiments; however, this treatment regimen had little effect on the concentration of serum IFN- γ in IL-12R $\beta 1^{-/-}$ allograft recipients.

Effects of IL-12 on alloantigen-specific Th1 development in vivo. Splenocytes obtained from cardiac allograft recipients were restimulated in vitro with donor alloantigens and supernatant concentrations of IFN- γ were determined by ELISA. This assay detects in vivo primed Th1, in that splenocytes from naive, nontransplanted mice produce minimal or undetectable levels of IFN- γ under these conditions (15, 16, 24). Restimulation of splenocytes from unmodified IL-12R $\beta 1^{-/-}$ allograft recipients with donor alloantigens resulted in the secretion of similar amounts of IFN- γ compared



Treatment In Vivo

FIGURE 3. Treatment of WT, but not IL-12R $\beta 1^{-/-}$ allograft recipients with rIL-12 markedly increases serum IFN- γ . WT or IL-12R $\beta 1^{-/-}$ C57BL/6 mice bearing BALB/c cardiac allografts were either left untreated or given daily intraperitoneal injections of $1.0 \mu\text{g}$ of rIL-12 on days 1–6 after transplantation. On day 7, blood obtained from allograft recipients was pooled and serum collected after centrifugation. Serum IFN- γ was determined by ELISA. Results are expressed as the mean cytokine concentration in triplicate samples \pm SD. Data are representative of three independent experiments. ND indicates not detectable. ***, $P < 0.005$ (WT IL-12-treated vs. IL-12R $\beta 1^{-/-}$ IL-12-treated).

to that seen in WT recipients ($\text{IL-12R}\beta 1^{-/-} = 7.16 \text{ ng/ml}$ vs. $\text{WT} = 6.24 \text{ ng/ml}$) (Fig. 4). Treatment of WT recipients with IL-12 resulted in a 10-fold increase in the production of IFN- γ (59.05 ng/ml). In contrast, IFN- γ production by splenocytes obtained from IL-12R $\beta 1^{-/-}$ recipients treated with IL-12 in vivo was similar to untreated values (9.59 ng/ml), indicating that the $\beta 1$ subunit of IL-12R is required for IL-12-mediated enhancement of in vivo sensitization of IFN- γ -producing cells. Further, these results indicate that in vivo Th1 development can occur in a state of IL-12 unresponsiveness.

Effects of exogenous IL-12 on cardiac allograft rejection. As IL-12 treatment markedly enhanced Th1 responses in WT allograft recipients (Figs. 3 and 4), one might predict that IL-12 treatment would exacerbate allograft rejection. To test this possibility, cardiac allograft function was monitored by daily abdominal palpation in WT or IL-12R $\beta 1^{-/-}$ allograft recipients bearing BALB/c hearts. Cardiac allograft recipients were either left untreated or injected once daily with $1.0 \mu\text{g}$ of rIL-12. Treatment of WT allograft recipients with this dose of rIL-12 ($n=10$) resulted in symptoms of cachexia including weight loss (mean decrease= $2.0 \pm 0.7 \text{ g}$ in 1 week), ruffed fur, hunched posture, and decreased activity. In contrast, IL-12R $\beta 1^{-/-}$ allograft recipients exhibited no signs of IL-12-induced toxicity.

The mean cardiac allograft survival in unmodified WT recipients was approximately 8 days (data not shown; 15, 24).

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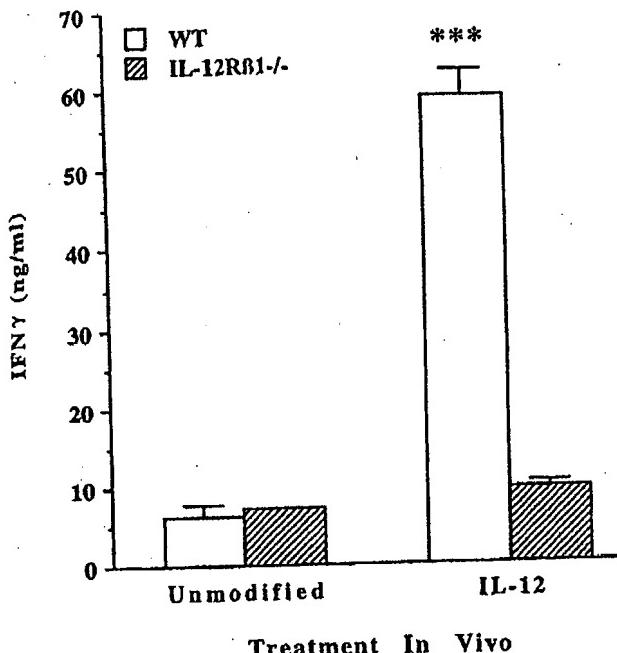


FIGURE 4. Enhancement of in vivo sensitization of IFN- γ -producing cells by IL-12 requires IL-12R β 1. WT or IL-12R β 1 $^{-/-}$ C57BL/6 cardiac allograft recipients were either left untreated or injected daily with 1.0 μ g of rIL-12 on days 1–6 after transplantation. To assess in vivo Th1 development, splenocytes (1×10^6 cells/ml) obtained from cardiac allograft recipients were restimulated with irradiated BALB/c splenocytes (1×10^6 cells/ml). Supernatants were collected after 72 hr, and the concentration of IFN- γ was determined by ELISA. Results are expressed as the mean concentration of IFN- γ in triplicate samples \pm SD. Data are representative of three separate experiments. ***, $P < 0.005$ (WT unmodified vs. WT IL-12-treated).

Cardiac allografts in IL-12R β 1 $^{-/-}$ recipients were rejected in a similar fashion to that seen in IL-12-deficient mice (15); in that grafts were uniformly rejected by day 7 ($n=8$). As expected, treatment of IL-12R β 1 $^{-/-}$ allograft recipients with rIL-12 had no effect on the tempo of allograft rejection ($n=6$). Interestingly, despite the overwhelming Th1 response induced by rIL-12 in WT allograft recipients (Figs. 3 and 4), treatment of these animals with rIL-12 did not appear to accelerate the tempo of graft rejection when compared to grafts of untreated WT recipients on day 7 after transplantation. For example, 7 of 10 (70%) allografts of WT recipients treated with rIL-12 were still functioning on day 7. A histologic evaluation of these grafts revealed similar parameters of early rejection compared to unmodified WT recipients. Specifically, histology was characterized by diffuse mononuclear cell infiltrates, viable myocytes as evidenced by visible nuclei, and relatively uninvolved vessels (Fig. 5). Hence, rIL-12 treatment did not accelerate the pathologic changes associated with acute rejection.

Phenotype of alloantigen-reactive Th1 in WT allograft recipients treated with rIL-12. To determine the phenotype of Th1 responsive to exogenous rIL-12, WT cardiac recipients were depleted in vivo of CD8+ T cells (Fig. 6). Splenocytes obtained from CD8 depleted cardiac allograft recipients produced markedly less IFN- γ upon in vitro restimulation with irradiated donor splenocytes (WT unmodified = 6.15 ng/ml vs.

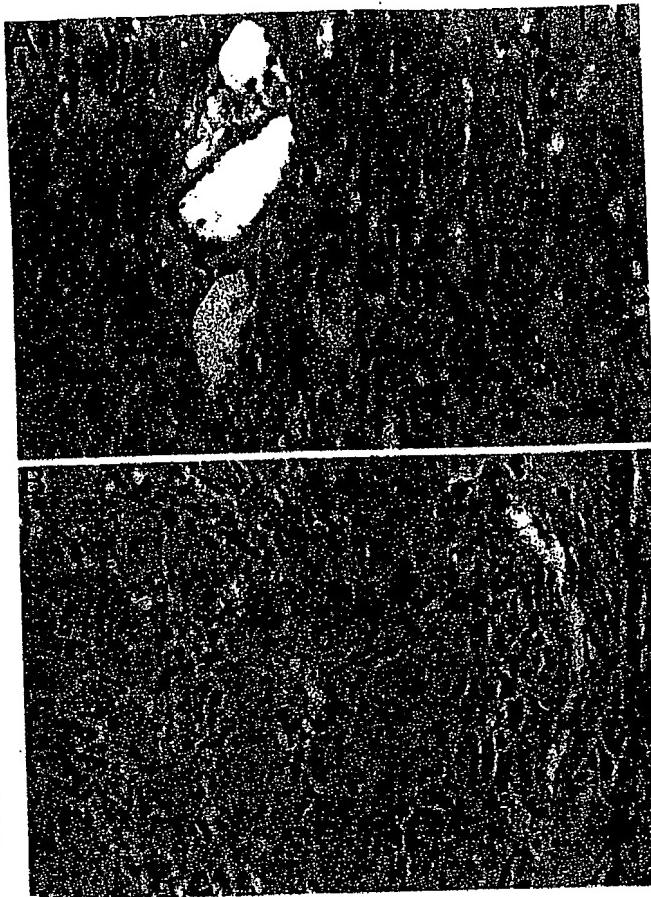


FIGURE 5. Exogenous IL-12 does not exacerbate cardiac allograft rejection. C57BL/6 WT recipients of BALB/c cardiac allografts were either left untreated or injected intraperitoneally with murine rIL-12 (1.0 μ g) on days 1–6 after transplantation. On day 7, allografts were harvested for histologic evaluation. (A) H&E-stained section of allografts from WT recipients left untreated (original magnification, $\times 400$). (B) H&E-stained section of allografts from WT recipients treated with rIL-12 (original magnification, $\times 400$). Note in both experimental groups moderate mononuclear cell infiltrates, and relative health of myocytes and vessels. These characteristics are associated with the early phase of acute rejection before onset of myocyte necrosis and vascular damage, which is observed on days 8 or 9 after transplantation. Results are representative of at least 10 individual transplants for each experimental group.

WT anti-CD8 mAb-treated = 0.52 ng/ml). Similarly, Th1 that develop as a result of IL-12 stimulation in these experiments were predominantly CD8+ T cells (Fig. 6), as depletion of CD8 cells resulted in a reduction in IFN- γ production (WT IL-12-treated = 35.89 ng/ml vs. WT IL-12 plus anti-CD8 mAb-treated = 2.75 ng/ml).

IL-12 Treatment (1.0 μ g/Day) Inhibits Alloantibody Responses

Given our findings that treatment of WT cardiac allograft recipients with rIL-12 resulted in significant augmentation of serum IFN- γ (Fig. 3) and in vivo priming of Th1 (Fig. 4), one would predict that IL-12 treatment should drive IgG2a alloantibody production. To test this possibility, sera alloantibody production was assessed on day 7 after transplan-

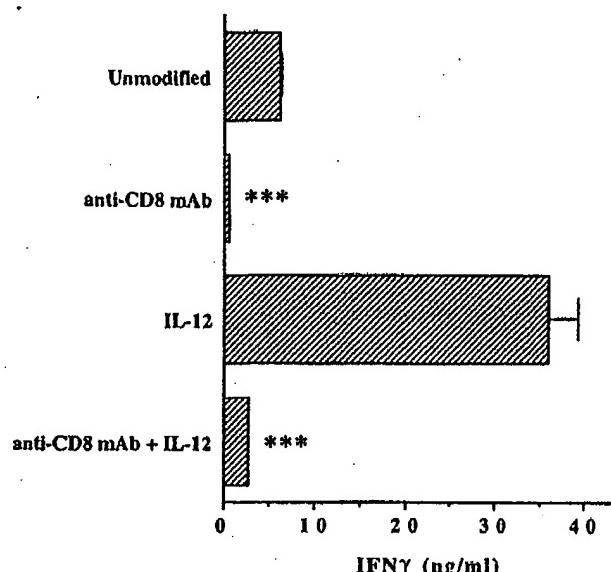


FIGURE 6. Alloantigen-specific Th1 responding to exogenous rIL-12 are CD8+ T cells. In these experiments, splenocytes were obtained from cardiac allograft recipients either left untreated or treated with anti-CD8 mAb, rIL-12, or a combination of anti-CD8 mAb plus rIL-12. Th1 function was assessed by IFN- γ production after a 72-hr restimulation of recipient's splenocytes with irradiated BALB/c splenocytes. Results are expressed as the mean concentration of IFN- γ in triplicate samples \pm SD. Data are representative of three separate experiments. ***, $P < 0.005$ (WT unmodified vs. WT anti-CD8 mAb-treated; WT IL-12-treated vs. WT IL-12 plus anti-CD8 mAb-treated).

tion in WT cardiac allograft recipients either left unmodified or treated once daily with 1.0 μ g of rIL-12. In these experiments, sera IgG2a was undetectable in rIL-12-treated WT allograft recipients at this time point (data not shown). Further, treatment of WT recipients with rIL-12 resulted in reduced sera IgM alloantibody in three independent experiments, compared to untreated WT recipients (Table 2). This observation indicates that high doses of rIL-12 inhibit, rather than enhance, alloantibody production in this model. In contrast, treatment of IL-12R β 1 $^{-/-}$ allograft recipients with

rIL-12 resulted in a slight increase in sera IgM compared to unmodified knockout recipients.

Effects of Low-dose IL-12 (0.1 μ g/Day) on Alloantibody Responses

The reduction in sera IgM in WT allograft recipients after rIL-12 (1.0 μ g/day) treatment (Table 2) may have been caused by anti-proliferative or toxic effects on B cell function caused by rIL-12 and/or IFN- γ . To test this possibility, WT cardiac allograft recipients were treated once daily with 0.1 μ g of rIL-12 and sera alloantibody levels were assessed on day 7 or 8 after transplantation. No alterations in the pathology of allograft rejection was observed in recipients treated with 0.1 μ g of rIL-12 compared to 1.0 μ g (data not shown). Further, treatment of WT allograft recipients with 0.1 μ g of rIL-12 resulted in significantly less IL-12-induced toxicity, serum IFN- γ concentrations in these animals were undetectable by ELISA, and in vivo Th1 sensitization was similar to untreated allograft recipients (data not shown). These results suggested that the 0.1- μ g dose of rIL-12 was ineffective in vivo. However, unlike the higher dose of rIL-12 (1.0 μ g), treatment of WT cardiac allograft recipients with 0.1 μ g of rIL-12 resulted in an increase in sera IgG2a, but not IgG1 at both days 7 and 8 after transplantation (Table 3). Further, IL-12 treatment augmented sera IgM levels at both time points.

DISCUSSION

Bioactive IL-12 exists as a p70 heterodimer composed of p35 and p40 subunits (28, 29). Each subunit of IL-12 interacts with a distinct component of the IL-12R: p40 binds to IL-12R β 1 and p35 interacts with IL-12R β 2 (19, 20). Both receptor subunits are associated with members of the Janus kinase family (30), and therefore may facilitate IL-12-mediated signal transduction. However, most of the attention has been given to IL-12R β 2, which associates with JAK2 (30). For example, recent evidence supports a requirement for IL-12R β 2 expression in IL-12-induced phosphorylation of Stat4 (21, 22). Further, these studies revealed the importance of the β 2 subunit of IL-12R by demonstrating that the unresponsiveness of Th2 to IL-12 in both human (21) and mouse (22) is a result of loss of IL-12R β 2 expression by these cells. These results indicate that the binding of the p40 subunit of

TABLE 2. High-dose rIL-12 inhibits IgM production in vivo^a

	Serum IgM (mean channel fluorescence)	
	Unmodified	IL-12 Treatment
WT		
Experiment 1	37.18	9.85
Experiment 2	33.97	11.39
Experiment 3	78.51	27.37
IL-12Rβ1$^{-/-}$		
Experiment 1	17.36	31.52
Experiment 2	33.83	43.64
Experiment 3	21.67	35.96

^a Serum was obtained on day 7 after transplantation from WT or IL-12R β 1 $^{-/-}$ cardiac allograft recipients. Animals were either left untreated or injected with 1 μ g of rIL-12 once daily. Anti-BALB/c IgM was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence and represent three separate experiments for each group.

TABLE 3. Low-dose rIL-12 augments IgM and IgG2a production in vivo^a

	Mean channel fluorescence		
	IgM	IgG1	IgG2a
Experiment 1 (day 7)			
Unmodified	11.90	2.06	2.58
IL-12	28.94	2.67	12.79
Experiment 2 (day 8)			
Unmodified	31.54	8.53	16.31
IL-12	46.57	9.72	49.00

^a Serum was obtained on day 7 or 8 after transplantation from cardiac allograft recipients either left untreated or injected with 0.1 μ g of rIL-12 once daily. Isotype-specific anti-BALB/c alloantibody was assessed by flow cytometry using P815 (H2^d) target cells as described under Materials and Methods. Data are reported as the mean channel fluorescence. Mean channel fluorescence for isotype controls were 1.65 (IgM), 1.67 (IgG1), and 1.57 (IgG2a).

IL-12 to IL-12R β 1 is not sufficient to mediate the bioactivity of heterodimer IL-12. However, we have reported that p40 promotes alloantigen-specific CD8+ Th1 development in the absence of heterodimer IL-12 (15). This observation suggests that IL-12 p40 mediates its stimulatory effect through IL-12R β 1 alone, or that IL-12R β 1 associates with a yet unidentified component of IL-12R on CD8+ T cells. These possibilities have not been tested. Additional data are emerging that support a biologic role of p40 interacting with IL-12R β 1 (1). Specifically, p35 knockout mice, which are capable of producing p40 in levels similar to WT mice (31), are less susceptible to infection with *Listeria* and *Cryptococcus neoformas* compared to p40 knockout mice. Hence, one goal of the current study was to assess the role of IL-12R β 1 in alloimmune responses both in vitro and in vivo.

IL-12 is a potent stimulator of in vitro alloantigen-specific Th1 development, in that the addition of IL-12 to MLC consisting of WT responder splenocytes resulted in a 10-fold or greater increase in IFN- γ production (Table 1; 15). Exogenous rIL-12 also markedly augments in vitro Th1 development in mice that are deficient in p35, p40 (15) or both subunits of IL-12 (JR Piccotti and DK Bishop, unpublished observations), indicating that T cells of these mice are equipped with a functional IL-12R. In contrast, IL-12 did not alter MLC IFN- γ production by splenocytes of IL-12R β 1 $^{-/-}$ mice (Table 1). This result illustrates the requirement of β 1 subunit of IL-12R for IL-12-driven Th1 differentiation in vitro. It should be noted that, although IFN- γ production by IL-12R β 1 $^{-/-}$ splenocytes in MLC was reduced compared to WT values (Table 1), this cytokine was readily detectable by ELISA, suggesting that IL-12 is not an absolute requirement for in vitro Th1 responses.

IL-12 is also a key cytokine involved in promoting cell-mediated immune responses in vivo (1, 2). However, what role IL-12 plays in transplant rejection remains unclear. It has been reported that IL-12 has a central role in the progression of acute graft-versus-host disease (GVHD) in mice (6, 32). In these studies, neutralizing IL-12 with a polyclonal anti-IL-12 antibody results in the amelioration of acute GVHD (32) and, conversely, treatment with exogenous IL-12 converts chronic GVHD into exacerbated acute GVHD (6, 32). Further, Williamson et al. (33) have reported that neutralizing IL-12 during the inductive phase of GVHD results in a Th1 to Th2 shift evidenced by a reduction in IFN- γ and enhancement of IL-5 and IL-10 production by Con A-stimulated splenocytes. In contrast to these findings, neutralizing IL-12 in mouse vascularized cardiac allograft recipients promotes intragraft Th2 cytokine (IL-4 and IL-10) gene expression; however, these grafts are rejected in an accelerated fashion compared to untreated recipients (16). Importantly, in vivo Th1 priming is not inhibited by IL-12 neutralization, indicating that Th1 development can occur independent of IL-12 (16). This possibility is further supported by the observation that splenocytes of IL-12R β 1 $^{-/-}$ allograft recipients produce similar concentrations of IFN- γ upon restimulation with donor splenocytes compared to WT recipients (Fig. 4). It does not appear that Th1 development in IL-12R β 1 $^{-/-}$ mice is a result of the interaction of endogenous IL-12 with the low-affinity IL-12R β 2, as treatment of these animals with rIL-12 did not augment in vivo priming of IFN- γ -producing cells (Figs. 3 and 4).

A second hypothesis tested in the current study was treatment of cardiac allograft recipients with IL-12 would accelerate the rejection process as a result of exacerbated Th1-driven immune responses. Administration of exogenous rIL-12 significantly augmented in vivo sensitization of IFN- γ -producing cells in WT cardiac allograft recipients, as evidenced by increased sera IFN- γ (Fig. 3) and enhanced production of IFN- γ by splenocytes after restimulation with donor alloantigens in vitro (Fig. 4). However, this fulminate Th1 response in vivo did not result in anticipated acceleration of graft rejection when compared to untreated control recipients (Fig. 5). It is possible that induction of high systemic levels of IFN- γ results in an inhibition of immune response as a result of IFN- γ 's anti-proliferative properties on effector cell development (34). However, graft survival was not prolonged after rIL-12 treatment in the current study. This observation questions the overall importance of Th1 responses in this experimental model, and suggests that the magnitude of Th1-driven alloimmune response may not correlate directly to the severity of graft rejection. Indeed, Th2-driven immune responses are emerging as potential effector cells of rejection in both human and experimental transplantation (reviewed in 35).

Finally, we examined the influence of rIL-12 administration on allospecific B cell function. In an experimental system in which PVG.RT1^a congenic rats were immunized with an isolated alloantigen, Gracie et al. (36) reported that treatment with murine rIL-12 (1.0 μ g/day for 5 days) after alloimmunization augments levels of allospecific IgG2b and IgG2c, while decreasing IgG1. The authors demonstrated that co-administration of neutralizing anti-IFN- γ mAb abrogated this response, indicating that the enhancement of B cell function by IL-12 was dependent on IFN- γ . When adjusted for body weight, this dose of rIL-12 in the rat is comparable to our 0.1- μ g dose in the mouse. In the current study, treatment of WT cardiac allograft recipients with 0.1 μ g of rIL-12/day for 6 days increased the level of sera IgG2a compared to untreated recipients on day 7 and 8 after transplantation (Table 3). However, administration of 1.0 μ g of rIL-12/day reduced allospecific B cell function, indicated by a decrease in sera IgM (Table 2) and absence of isotype switch to IgG2a. These observations suggest a biphasic response to IL-12 treatment in WT mouse cardiac allograft recipients likely dependent on the concentration of IFN- γ .

In summary, this study illustrates that the β 1 subunit of mouse IL-12R is critical for IL-12-driven alloimmune responses both in vitro and in vivo, and that IL-12R β 2 alone does not transduce IL-12 signaling. These observations are supported by recent reports, which have shown that humans deficient in IL-12R β 1 exhibit severe impairment in their resistance to infections as a result of intracellular pathogens (37, 38). The generation of mice deficient in IL-12R β 2 will provide an important animal model to evaluate whether β 1 subunit of IL-12R alone conveys IL-12 responsiveness in vivo. Specifically, these mice would be useful in determining the mechanism by which p40 subunit of IL-12 enhances CD8+ Th1 development (15, 16). Finally, this study questions the importance of Th1-driven alloimmune responses in cardiac allograft rejection, as exacerbated Th1 responses induced by IL-12 failed to accelerate graft rejection in this model.

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Immunosuppressive therapy

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Although Cyclosporin A has improved transplant outcome, its use has serious limitations due to its narrow therapeutic window. New approaches to broaden this window exploit alternative drug formulations, pharmacokinetic profiling and new immunosuppressive agents, such as Rapamycin and Brequinar, which act in a synergistic fashion. There is no evidence to suggest that the pharmacological alternative to Cyclosporin A, FK-506, displays a broader therapeutic window, although it may be tenfold more potent. Similarly, despite the specificity of the IgG2a mouse anti-human CD3 monoclonal antibody, it displays a significant range of clinical side effects, delayed therapeutic action and frequently stimulates generation of human anti-mouse monoclonal antibodies. Recent advances in monoclonal antibody technology seek not only to produce antibodies against determinants involved in alloactivation, but also to 'humanize' the antibodies for reduced side effects. The availability of this array of potential agents highlights the need to develop guidelines for clinical trial methodologies to address the unique needs and demands of organ transplantation.

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Introduction

After thirty years of vigorous but relatively unproductive research, the field of immunosuppressive drugs awakened following the approval of two agents that, in contrast to the non-selective drugs Azathioprine (Aza) and corticosteroids, display relatively specific actions on T cells. One of these, the fungal undecapeptide Cyclosporin A (CsA), not only improved clinical outcomes and broadened the clinical settings in which transplants were successful, but also provided a unique tool for dissecting activation mechanisms leading to lymphokine synthesis. Subsequent approval of the other agent, the IgG2a mouse monoclonal antibody (mAb) OKT-3, heralded the use of reagents that bind selective T-cell surface markers to modulate the immune response. The past decade has witnessed striking progress in the development of new pharmacological agents (Fig. 1). One group inhibits lymphokine biosynthesis, FK-506 or signal transduction, Rapamycin (RAPA). A second group is the nucleotide synthesis inhibitors; Mizoribine [1] and RS61443, a morpholinoethyl-ester analog of mycophenolic acid (MPA) [2**], block purine salvage pathways with the generation of guanosine monophosphate, and the quinoline carboxylic acid Brequinar (BQR) blocks the *de novo* synthesis of pyrimidines [3**]. A third group,

new mAbs, recognizes specific surface epitopes on T cells and antigen-presenting cells (Fig. 2). Immunosuppressive activity has been documented [4-9] with several mAbs that bind various determinants as shown in Table 1. Prolonged graft survival has also been achieved with antibodies, or preferably their F(ab')₂ fragments, directed towards class I [10] or class II MHC antigens. A refinement of mAb technology is the production of immuno-toxins. Ricin α-chain toxin linked to mouse anti-human CD5 IgG1 mAb has been used by Haverty (personal communication) to treat steroid-resistant graft versus host disease in human bone marrow transplantation. This array of new agents proffers an unprecedented opportunity to design effective, yet minimally toxic, regimens to improve the outcome of transplantation in man.

Limitations of existing immunosuppressive regimens

Currently, clinical regimens are based upon the use of CsA, the immunosuppressant benefits of which are seriously limited by side effects. In attempts to augment its efficacy, the corticosteroid Prednisone (Pred), Aza,

Abbreviations

- ALG—anti-lymphocyte sera; Aza—Azathioprine; BQR—Brequinar; CMV—cytomegalovirus; CsA—Cyclosporin A; CTL—cytotoxic T lymphocyte; DTH—delayed type hypersensitivity; ICAM—intercellular adhesion molecule; IL—interleukin; LFA—lymphocyte function-associated antigen; mAb—monoclonal antibody; MHC—major histocompatibility complex; MPA—mycophenolic acid; MZB—mizoribine; NF-AT—nuclear factor of activated T cells; Pred—Prednisone; RAPA—Rapamycin; TCR—T-cell receptor; Th—T-helper.

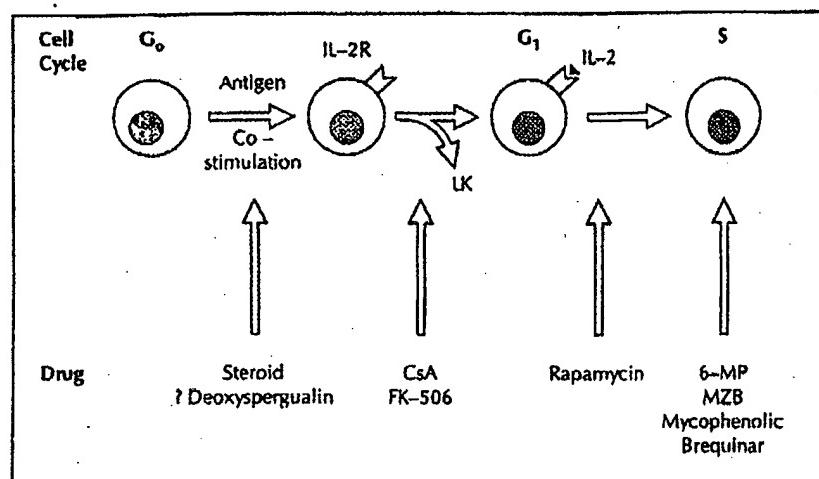


Fig. 1. Classification of immunosuppressive drugs based upon their site of action in the cell cycle. In the first group, corticosteroid, and possibly deoxyspergualin, inhibit antigen-presenting cells. In the second group, Cyclosporin A (CsA) and FK-506 inhibit lymphokine (LK) biosynthesis during the G₀ phase and Rapamycin inhibits signal transduction during the G₁ phase. In the third group (the nucleoside synthesis inhibitors), Mizoribine (MZB) and RS61443 (a morpholinoethyl-ether analog of mycophenolic acid, MPA) inhibit purine synthesis pathways leading to the generation of guanosine monophosphate, whereas Brequinar (quinoline carboxylic acid) inhibits the de novo synthesis of pyrimidines. IL, interleukin; R, receptor.

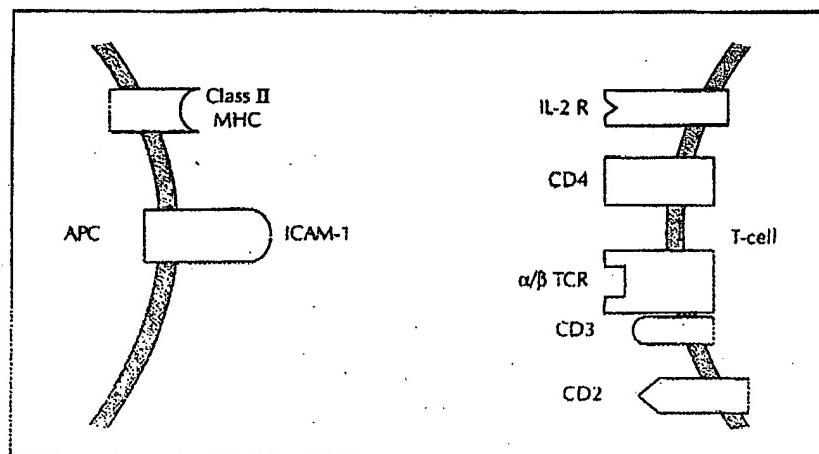


Fig. 2. Epitope targets of monoclonal antibodies. ICAM, intercellular adhesion molecule; IL, interleukin; MHC, major histocompatibility complex; R, receptor; TCR, T-cell receptor.

Table 1. Determinants recognized by immunosuppressive monoclonal antibodies

Monoclonal antibody	Determinant	Reference
IgM T10B9.1A-31	Determinants common to α/β chains of all human T-cell receptors	[4]
IgG2b BMA 031		[5]
OKT4A	CD4	[6]
SDZ CHH 380	CD7	[7]
33B3.1	α chain or α/β complex of interleukin-2 receptor	[8]
BIRR-1	Intercellular adhesion molecule-1 (or CD54)	[9]
25.3	Lymphocyte function-associated antigen-1	

equine or rabbit polyclonal anti-lymphocyte sera (ALG) and/or mouse OKT-3 mAbs have been combined in empirical regimens that are often tailored to individual patients. To address one limitation of CsA, nephro- and/or hepato-toxicity during the initial post-transplant phase of induction immunosuppressive therapy, which delays allograft and patient recovery, pharmacokinetic control

programs have been used either to pre-select CsA doses [11] or to combine ALG or OKT-3 with Aza/Pred in order to delay treatment with CsA. However, immediate, rather than delayed, administration of CsA to patients displaying good initial renal function avoids the disadvantages of ALG/OKT-3 induction, namely an increased risk of cytomegalovirus (CMV) infection, additional expense and/or delayed hospital discharge awaiting satisfactory CsA levels after treatment with CsA. However, patients at extraordinarily high immunological risk, due to rejection of previous allografts within three months, or with marginally-functioning organs may preferably be treated with ALG or OKT-3 induction as a possible means to delay the onset of their first rejection episodes. Two possible alternatives for induction therapy are the CsA analogs that may display reduced nephrotoxicity, namely Cyclosporin G [12], which substitutes norvaline at position 2, and IMM-125 with a hydrophilic substituent at position 9.

To date, no large randomized study has shown that the induction regimen alters the clinical outcome; rather, a small cohort reported by Belitsky *et al.* [13] showed no difference between ALG versus initial CsA therapy, the two options for induction therapy. There may be several reasons for this. Firstly, the polyclonal reagents, such as

rabbit or equine Minnesota ALG and anti-thymocyte globulin, regardless of their source, opsonize T cells, leading to their removal from the circulation. This depletion obviates T-cell mediated attack on the allograft. Among other factors that must be considered is that the central intravenous lines required for polyclonal administration may be accidentally contaminated, producing septicemia. Further, there are no indices of the efficacy of the polyclonal sera. Peripheral blood T-cell numbers above the target range of 50–150/ml tend only to be useful reflections of the appearance of human host anti-equine antibodies, not immunological resistance to therapy. The other induction therapy using OKT-3, which covers and/or modulates CD3 epitopes on the T-cell surface, offers the advantages of both peripheral intravenous administration and readily available fluorescence-activated cell sorting (FACS) tests for antibody efficacy. The clinician monitors patient peripheral blood lymphocyte T-cell epitopes for (a) cells with exposed CD3 epitopes that were not bound by OKT-3 *in vivo* by their capacity to bind fluorescinated OKT-3 *in vitro*, (b) the total number of circulating T cells with fluorescinated anti-CD2, a pan T-cell marker, and (c) the proportion of OKT-3-coated cells detected with a goat anti-mouse IgG reagent. A satisfactory therapeutic effect is observed when the patient has <25% OKT-3⁺ cells *in vitro* and 60–75% CD2⁺T cells, about 40% of which are coated with mouse IgG which binds to OKT-3. While OKT-3 represents an advance in immunosuppressive therapy, it has several serious limitations: (a) severe first-dose reactions, including chills, fever, myalgias and, in the worst cases, pulmonary oedema apparently due to lymphokine release (particularly tumor necrosis factor and interleukin (IL)-2); (b) longer-term adverse effects such as aseptic meningitis; (c) a delay in the therapeutic effect for as long as 7 days after initiation of treatment; (d) induction of human anti-mouse antibodies, generally of the anti-idiotype variety, but not uncommonly of broader reactivity; (e) a frequent incidence of rebound re-rejection episodes upon completion of the therapeutic course; and (f) a tendency toward CMV infections in 40% of treated patients. In addition, both polyclonal and mAb reagents may produce excessive immunosuppression, resulting in increased incidences of CMV infection and/or of lymphomas and other neoplasms, as well as allograft thrombosis. Thus, selection of a CsA versus an antibody induction regimen must balance the risks of nephrotoxicity versus that of excessive immunosuppression.

The use of CsA has reduced the risk of acute rejection, but a rational approach to CsA administration is confused by the tremendous variability between individuals in drug pharmacokinetics and pharmacodynamics [14], which, in turn, generates a fear of irreversible renal injury in case the CsA dose is excessive. Three approaches have been used to address this problem: (a) combining reduced CsA doses with subtherapeutic amounts of Aza [15]; (b) monitoring the parent compound CsA based upon its trough concentration prior to the next drug dose [16]; and (c) adjusting CsA doses prospectively based upon average concentrations calculated from serial measurements of the area under the concentration-time curve

[11]. Since drug absorption presents the greatest variability in pharmacokinetics, attempts have been made to increase CsA bioavailability by co-administration of Vitamin E [17]. In addition, the manufacturer has produced a new micro-emulsion formulation that increases the bioavailability by twofold above that of the existing oral solution or capsule preparation, which show equivalent bioavailability in studies comparing both formulations [18]. The critical issue seems to be the drug concentration in the allograft. While direct intra-arterial infusion has been used for experimental models of renal or cardiac allografts, good drug uptake in man can be achieved by presenting CsA as an aerosol in absolute ethanol when it has a mean particle diameter of 1.2 microns [19].

Optimal use of CsA demands the measurement of drug concentrations/activities at the level of its lymphocyte receptor or target signal transduction molecule(s), which may be calcineurin (an enzyme that may be involved in a common step associated with T-cell and IgE receptor signaling pathways) [20•] or the nuclear factor of activated T-cells (NF-AT). However, a major limitation may be the failure of CsA to inhibit lymphocyte activation via the CD28 surface marker [21], an important co-stimulatory pathway that together with T-cell receptor (TCR) stimulation blocks induction of anergy in T-cell clones [22]. Fortunately, rejection episodes under CsA prophylactic therapy tend to be readily reversed by corticosteroid therapy, and the majority of steroid-resistant episodes are overcome with polyclonal ALG and/or OKT-3 therapy. Corticosteroids are believed to represent the Achille's heel of transplantation because of the wide distribution and pleiotropic effects of the glucocorticoid receptor superfamily found in the cytoplasm. These are DNA-binding dimeric transcription factors with a zinc finger structure that recognize enhancer (or negative regulator) elements bearing the GRE motif (GTACAnnnT-GTTCT, where n = any nucleotide). One important negative regulatory element is the AP-1 binding site, normally the focus for fos-jun heterodimers [23]. An alternative approach to the reduction of IL-1 β generation, an action typical of corticosteroids, is to inhibit the enzyme that cleaves the inactive 31 kD precursor between Asp¹¹⁶ and Ala¹¹⁷ to release the 153 carboxyl-terminal amino acids that constitute IL-1 β [24]. Another immunosuppressive effect may be achieved by the upregulation of the synthesis of transforming growth factor- β by steroids [25]. Withdrawal of steroid treatment months to years after the transplant may be successful in patients who did not reject the transplant [26•].

Preliminary data suggest that a ten-day course of the IgM mouse anti-human α/β TCR mAb T10B9.1A-31 [4], but not BMA031 (C Groth, personal communication), not only produces equivalent therapeutic effects to those of OKT-3, but is less toxic in terms of incidence of fever and neurological and respiratory symptoms, as well as of subsequent infections. Furthermore, T10B9 therapy is not associated with as great a rise in serum creatinine during treatment as OKT-3, suggesting a more rapid attenuation of the allo-immune response. However, the repeated use of xenogeneic antibodies during the induc-

tion phase as well as for anti-rejection therapy may be complicated by the development of neutralizing human anti-mouse antibodies.

A major goal of maintenance immunosuppressive therapy is prophylaxis against chronic rejection. To date, not only has CsA/Pred therapy failed to reduce the incidence of this complication from the 8–10% level observed under the Aza/Pred combination, but there is no way to determine if the failure is due to its inherently modest inhibition of B-cell responses or to physicians' tendency to limit CsA therapy to minimal, possibly ineffective, doses in order to mitigate a renal injury. Thus, despite the improvement in initial graft survival, transplants continue to be lost in the longer term, with half-lives of about seven years for cardiac and 11.5 years for renal transplant in humans. A recent study of the effects of immunosuppressive drugs on coronary vascular disease in heterotopic rat cardiac allografts suggests that RAPA particularly, CsA to a lesser extent, but definitely not FK-506, inhibit pathological endothelial and smooth muscle lesions in arteries and arterioles, which seem to be the critical lesions in the progression of chronic rejection [27].

New pharmacological agents

Both the macrolide FK-506 and the undecapeptide CsA interrupt lymphokine synthesis by inhibiting generation of the Ca^{2+} dependent regulatory proteins NF-AT, NFIL-2A, NFIL-2B, and NF- $\kappa\beta$, but not c-fos, which is necessary for IL-2 generation. Presumably, both drugs also affect serine protease gene transcripts, an excellent marker of rejection [28]. The inhibition of cytotoxic T lymphocytes (CTLs), even in the presence of optimal amounts of IL-2, is a prominent effect of CsA [29] and, apparently, FK-506. Despite the assumption that CsA and FK-506 produce similar inhibitory effects, at least three differences have been observed: first, FK-506 displays a flatter inhibition curve than CsA with a wider discrepancy in potency at the 50% inhibition than at the 95% inhibition level; secondly, CsA leads to the generation of suppressive T cells, whereas FK-506 does not; and thirdly, although both drugs inhibit $\text{CD}4^+$ T helper (Th) lymphocytes, which secrete IL-2, only CsA (and not FK-506) permits priming of $\text{CD}8^+$ CTLs [30]. Furthermore, Bretscher and Havelé [31] suggest that CsA switches the immune response to the graft from a delayed type hypersensitivity (DTH) response to an IgG response by inhibiting the Th1 subset with the emergence of the Th2 subset, which actively induces IgG via IL-4 generation and inhibits Th1 cells and DTH via IL-10. Both CsA and FK-506 spare transcription of the down-regulatory lymphokine IL-10. While CsA inhibits transcription of IL-6, this factor is not affected by FK-506.

The coming year should witness publication of a vast array of randomized trials comparing the clinical outcome of liver and renal transplants in patients treated with FK-506 versus CsA. So far, a preliminary non-randomized study of liver recipients showed that FK-506 therapy displays greater neurotoxicity, equivalent nephrotoxicity,

but, possibly, less hypertension than does CsA therapy [32**]. A further claim that corticosteroids do not have to be used with FK-506 cannot be assessed due to two factors: firstly, the protocol stipulated higher Pred doses in the CsA cohort than those used with FK-506; and secondly, to date, there is no pharmacokinetic analysis of Pred concentrations in CsA versus FK-506 treatment groups in order to exclude a drug interaction. Additionally, the extremely poor results in the initial study, wherein allegedly CsA-resistant patients were converted to treatment with FK-506, actually reflected antagonism between the two drugs caused by (a) an adverse immunological interaction between the two agents that apparently have similar mechanisms of action [33] and (b) competitive pharmacokinetic interactions. Although FK-506 has not yet been shown to achieve clinical results even equivalent to those of CsA, eventual definition of its relative therapeutic window will depend upon Phase II studies to select well-tolerated drug doses for randomized trials versus CsA therapy.

When a second agent, RS61443, was added in doses of 2 500–3 500 mg per day to a CsA/Pred regimen, it seemed to reduce the incidence of acute rejection episodes. However, these high doses are likely to produce toxicity, particularly leukopenia and gastrointestinal complaints [2**]. Randomized placebo-controlled trials are underway to assess the efficacy of RS61443 versus Aza added to a CsA/Pred regimen. Other studies are examining the impact of a fourth agent, deoxyspergualin, to potentiate an ALG/Aza/Pred/CsA induction protocol.

The studies that claimed Aza displays pharmacological synergism with CsA failed to utilize rigorous experimental design or data analysis [34]. For instance, both *in vitro* analyses [35] and clinical results demonstrate that Aza acts in an additive manner rather than synergistically with CsA [36]. Similarly, *in vitro* analyses suggest that RS61443 [37], mizoribine [37], and thalidomide [38] also act in an additive manner with CsA. Although initial data suggested that BQR potentiates the effect of CsA [3**], recent experiments document true synergism [37]. However, CsA/RAPA combinations show the most impressive degree of synergy both *in vitro* and *in vivo* [39]. Once Phase I toxicity trials have been completed, it will be possible to assess whether BQR or RAPA displays the synergistic effects with CsA in human transplantation that are evident in rodents and large animal models.

New monoclonal antibody reagents

Second generation mAbs are being designed to avoid the severe systemic reactions due to lymphokine release that follow initial doses of OKT-3. For example, the IgG2b anti-human α/β TCR mAb BMA 031 used for induction therapy (three 50 mg doses administered on alternate days) delays the onset of first rejection episodes and probably improves one-year graft survival (R Knight and BD Kahan, unpublished data). Similar benefits have been reported with mouse and rat mAbs produced against the activation-induced α -chain, or to new epitopes resulting

from the formation of the $\alpha\beta$ complex, of the IL-2 receptor [8].

However, treatment with these antibodies leads to a high incidence of human anti-mouse antibodies, which may attenuate the immunosuppressive effects. Recent work has explored approaches to construct either (a) 'chimeric' antibodies bearing human Fc segments joined to mouse $F(ab')_2$ fragments, or (b) 'humanized' mAbs with mouse idiotypes inserted onto human IgG isotypes (Fig.3). Chimeric antibodies combine the variable regions of mouse antibodies with human antibody constant regions and, therefore, present fewer foreign amino acid sequences to the host. However, one-third of the structure is still of mouse origin. Furthermore, a clinical trial using a chimeric anti-CD7 mAb not only failed to achieve a superior level of immunosuppression induction, but also increased the incidence of vascular thromboses [7]. The latter effect may have been related to the adhesion of Fc receptors on platelets and polymorphonuclear leukocytes to the human Fc regions, bound to endothelium via mouse epitopes. On the other hand, 'humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen combining site, with human variable region frameworks and constant regions. Due to the reduced affinity of 'humanized' antibodies for antigen epitopes, Co *et al.* [40•] recommended two innovations: firstly, selection of a human framework that is as homologous to the original mouse antibody as possible; and secondly, insertion of key residues from the mouse model into the construct in order to achieve a molecular conformation that is similar to the native idiotype. The beneficial effects of chimeric and 'humanized' variants of mouse mAbs will be clarified only by randomized clinical trials.

Two alternative approaches seek to utilize mAbs directed against donor MHC antigens or against co-receptor molecules. In a study of non-human primates,

OKT-4A IgG2A mAbs, which react with the CD4 co-receptor on Th cells, provoked fewer side effects than OKT-3 [6]. In an initial clinical trial of OKT-4A induction therapy (0.2 mg/kg/day), all six patients suffered rejections. These rejections were reversible, but left residual areas of dead tissue resulting from an obstruction of the blood supply in half the renal allografts. Unfortunately, OKT-4A also generated a strong human anti-mouse antibody response (J Barry, personal communication). Experimental animal models are currently being used to determine if antibody efficacy is related to T-cell deletion and is potentiated by simultaneous treatment with an anti-CD8 mAb. On the one hand, Fathman and colleagues [41] found that depleting anti-CD4 mAbs produced prolonged allo-unresponsiveness toward allogeneic pancreatic islets, an effect that was moderated by simultaneous treatment with anti-CD8⁺ mAbs, suggesting the role of a regulator CD8⁺ cell. On the other hand, Waldmann and colleagues [42] induced tolerance toward mouse heart transplants where the donor and recipient were not matched at MHC level using an anti-CD4 mAb that not only did not deplete T cells but also was potentiated by simultaneous administration of an anti-CD8 mAb.

A second approach to co-receptor molecules is based on the interaction of lymphocyte function-associated antigen (LFA)-1 on CTLs with the intercellular adhesion molecule (ICAM)-1 or monocytes. Expression of ICAM-1 is up-regulated following lymphokine release, which occurs during acute allograft rejection but not during other pathological events in the kidney [43**]. Prophylactic and therapeutic administrations of a mAb directed against the high molecular weight α -chain of human ICAM-1 alone delayed both the onset and progression of rejection episodes in primate renal allograft models. Using mouse mAbs directed against LFA-1, Stoppa *et al.* [44] reversed steroid resistant acute graft versus host reactions in man. Indeed, the combination of anti-ICAM-1 and anti-LFA-1 mAbs produced allo-tolerance in mice that were not com-

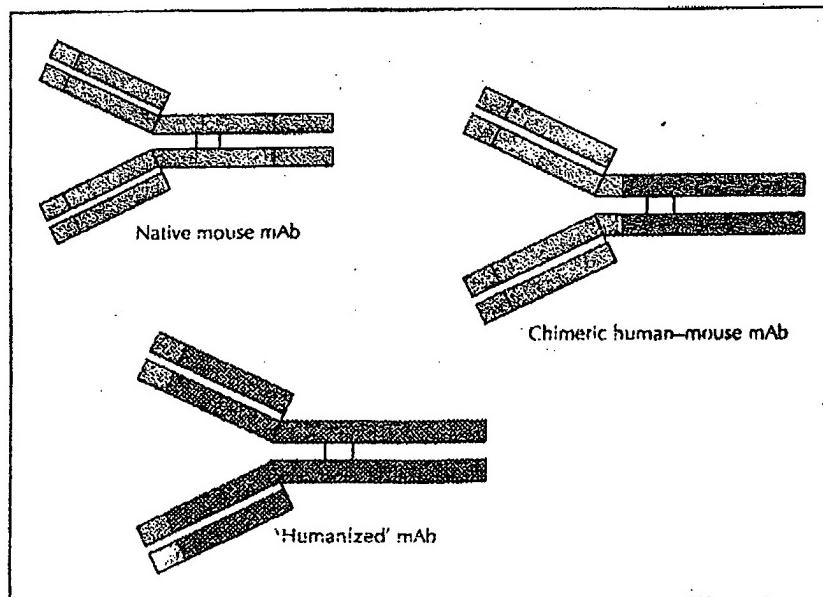


Fig. 3. Types of monoclonal antibody. Chimeric antibodies combine the variable regions of mouse antibodies with human constant regions and, therefore, present fewer foreign amino acid sequences to the host. 'Humanized' antibodies combine only the smallest part of a mouse antibody that is required, the antigen binding site, with human variable region frameworks and constant regions.

patible at the MHC level [45••]. These promising results in animal models using mAbs directed against T-cell and monocyte co-receptors await confirmation in controlled clinical trials.

While clinical interventions to date have focused on using mAbs directed towards surface epitopes important for the afferent limb of the allo-immune response, there is increasing evidence that anti-idiotypic antibodies, either exogenously introduced or endogenously, spontaneously generated, may regulate the induction of allo-immune responses. A recent study performed by Snider [46] suggested that immunization of hosts with antigen-antibody complexes confers a bias in the epitope, resulting in a less efficient antibody response that shows anti-idiotypic properties. This approach represents a particularly fertile ground for clinical exploration.

Cytokine receptor analogs and antagonists

A new group of immunosuppressive agents are the cytokine receptor antagonists. The discovery and initial testing of an IL-1 receptor antagonist has been reviewed by Arend [47]. IgG-stimulated human monocytes naturally produce IL-1 receptor antagonist, a heterogeneous array of glycoproteins of 15–25 kD, depending upon their degree of glycosylation. IL-1 receptor antagonist binds type I, but not type II, IL-1 receptors without activating cells and with considerably less avidity than native IL-1 α and IL-1. Type I IL-1 receptors are present on Th2 cells and fibroblasts; Type II IL-1 receptors are present on B cells, neutrophils, and macrophages. Although therapeutic trials of IL-1 receptor antagonist in rheumatoid arthritis and septic shock suggest some beneficial effects, Faherty *et al* [48] failed to observe that IL-1 receptor antagonist inhibited induction of CTLs, cutaneous DTH, or T-cell dependent humoral antibody responses. They also found that administration of a mAb to type II IL-1 receptor (35F5) was ineffective. Fanslow *et al* [49•] recently extended their previous studies, which used constructs of the extra-membranous portion of the IL-1 receptor, by using similar constructs of the IL-4 receptor. In the initial studies, they prolonged heterotopic pinnal, neonatal mouse heart allograft survival, but failed to prevent allo-sensitization, as documented by a rapid, secondary-type proliferative response upon *in vitro* one-way mixed lymphocyte reactions. In their recent studies, constructs of IL-4 receptor alone, or in combination with rat anti-mouse IL-4 receptor mAb, induced modest prolongation of heart allo-explants.

Immunosuppressive drug trials

Of the numerous obstacles currently hindering the development of efficacious immunosuppressive regimens, the lack of methodology for clinical transplantation trials is of particular importance. To date, no series of Phase I and II toxicity and dose-finding trials has been conducted in

order to establish a foundation for clinical investigation. The introduction of Aza, steroids, and CsA, as well as the preliminary trials of FK-506, have relied upon empirical approaches. Important obstacles to comprehensive trials include the relatively small numbers of transplant cases, the use of unrefined end-points such as graft and patient survival, and the lack of well-established criteria for the diagnosis and grading of rejection episodes, deficits that obfuscate the use of this event as an intermediate endpoint. In addition, no *in vitro* immune assay predicts or correlates with *in vivo* immunosuppressive efficacy; hence, there is no surrogate immune parameter as a basis of immunosuppressive efficacy and/or for dose extrapolation from *in vitro* systems to *in vivo* conditions.

Since present results with CsA-based regimens yield excellent graft survivals, extremely large numbers of patients must be entered into clinical trials to document improved efficacy of a new agent. Even more extensive efforts will be needed to exclude the possibility that the results with the new agent are not actually worse than those obtained with the existent CsA regimen. In light of the presently high success rates, the benefits of any new regimen must be based upon both the potency and the mitigation of side effects, as assessed by quantitative parameters, including glomerular filtration rates. The practice of clinical research in transplantation must proceed to develop principles of rigorous study design and precise analytical tools in order to most expeditiously evaluate the available array of new immunosuppressants described in this review.

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Differential effects of IL-12 on the generation of alloreactive CTL mediated by murine and human dendritic cells: a critical role for nitric oxide

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Abstract: We examined the mechanisms involved in interleukin (IL)-12-mediated suppression of cellular immunity in mice using allogeneic mixed leukocyte reaction (MLR) stimulated by dendritic cells (DCs) *in vitro* and compared the effect of IL-12 on MLR in mice and humans. Although IL-12 stimulated human MLR, the addition of IL-12 or interferon- γ (IFN- γ) resulted in a dose-dependent suppression of MLR in mice. The treatment with N^G-monomethyl-L-arginine (L-NMMA) completely abrogated IL-12- and IFN- γ -mediated suppression of MLR in mice. Furthermore, IL-12 enhanced the alloreactive cytolytic T lymphocyte (CTL) induction in human MLR, whereas the addition of L-NMMA was required to generate alloreactive CTLs in the presence of IL-12 in mice. Nitric oxide (NO) was detected only in mouse MLR. Murine DCs could produce NO, but neither human CD34⁺ cell- nor monocyte-derived DCs produced a detectable amount of NO. These results suggest that NO produced by DCs might play an important role in IL-12-mediated immune suppression in mice but not in humans. *J. Leukoc. Biol.* 73: 621–629; 2003.

Key Words: MLR · IFN- γ · L-NMMA

INTRODUCTION

Interleukin (IL)-12 is a heterodimeric cytokine produced by dendritic cells (DCs), macrophages, polymorphonuclear leukocytes, and keratinocytes [1]. Pleiotropic effects on the host immune system, including activation of natural killer (NK) cells and cytolytic T lymphocytes (CTLs), induction of interferon- γ (IFN- γ) production and T helper cell type 1 (Th1) differentiation, and antiangiogenic effects [1–4], have been reported. Exogenous IL-12 is effective for infectious and malignant diseases [1–6]. Furthermore, IL-12 gene transduction into tumors strongly suppressed the growth of various tumors *in vivo* [7, 8], whereas the mechanisms involved in the antitumor

effects of IL-12 are still unclear as a result of the complexity of the biological effects of IL-12.

Conversely, the unexpected effects of IL-12 on the cellular immune responses have been reported in mice. Noguchi et al. [9] showed that administration of a low dose of IL-12 (1 ng/mouse) enhanced CTL activity specific for the mutated p53 peptide (234CM) and regressed the MethA tumor expressing it, whereas a high dose of IL-12 (100 ng/mouse) suppressed 234CM-specific CTL generation. Martinotti et al. [8] reported that a CD8⁺ T cell-mediated *in vivo*-immune response against IL-12-transduced colon cancer cells was observed only when CD4⁺ T cells were abrogated. Furthermore, Orange et al. [10] reported that administration of IL-12 decreased antiviral CTL activity in the lymphocytic choriomeningitis virus (LCMV) infection model. The report presented by Piccotti et al. [11] demonstrated that IL-12 antagonism using anti-IL-12 antibodies (Ab) or an IL-12 p40 homodimer exacerbated cardiac allograft rejection in mice. Recently, Kurzawa et al. [12] clearly demonstrated the suppressive effects of IL-12 on cellular immune responses using syngeneic and allogeneic murine tumor systems. Further studies reported by Koblish et al. [13] clarified that nitric oxide (NO) produced by macrophages was responsible for IL-12-mediated immune suppression in mice *in vitro* and *in vivo*. On the basis of these results, they suggested the monitoring of suppressive effects of IL-12 when used for malignant and infectious diseases in humans.

DCs are known to be specialized antigen-presenting cells (APCs), which exist in virtually every tissue, capture antigens *in situ*, and migrate to lymphoid organs to activate naive T cells and play a key role in the induction of primary immune responses [14]. As murine DCs are also known to be the producer of NO [15], we examined whether NO derived from DCs is a suppressive factor for the cellular immune responses in allogeneic mixed leukocyte reaction (MLR) stimulated by IL-12 in mice and humans. It is interesting that a high dose of

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IL-12 suppressed alloreactive proliferation of T cells in mice but enhanced it in humans. Furthermore, we found the high production of NO by murine DCs, whereas neither human CD34⁺ progenitor cell- nor monocyte-derived DCs produce a significant amount of NO even after stimulation with IFN- γ and/or lipopolysaccharide (LPS) as well as MLR. These results suggest that immune responses mediated by IL-12 in mice are, at least in part, different from that in humans and that the suppressive effect of IL-12 on cellular immune responses might not be the case in humans.

MATERIALS AND METHODS

Reagents

The culture media was RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 μ g/ml streptomycin, 100 U/ml penicillin, and 5.5×10^{-5} M 2-mercaptoethanol (all from Life Technologies, Grand Island, NY), referred to henceforth as complete medium (CM). The recombinant mouse and human (rh)IL-12 was kindly provided by Dr. Maurice Gately (Hoffmann-La Roche, Nutley, NJ). The rhIFN- γ was purchased from Boehringer Mannheim (Indianapolis, IN). Specific activities were $>5.0 \times 10^6$ U/mg and $>2.0 \times 10^7$ U/mg, respectively. N^G-monomethyl-L-arginine (L-NMMA) and NO-generating agent S-nitroso-N-acetyl-penicillamine (SNAP) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines and mouse strains

EJ-4 and P815 were generous gifts of William H. Chambers (University of Pittsburgh, PA). These cell lines were maintained in CM as described previously [7].

Female 6- to 8-week-old C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). Female 6- to 8-week-old BALB/cj mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These animals were used for all experiments at the age of 8–10 weeks old [7].

Culture of mouse bone marrow (BM)-derived DCs

BM-DC culture was obtained using methods described previously [16–19]. Briefly, murine BM cells were harvested from the femur and tibia of killed mice. Contaminating erythrocytes were lysed with 0.83 M NH₄Cl buffer, and lymphocytes were depleted with a cocktail of antibodies (RA3-3A1/6.1, anti-B220; 2.43, anti-IgT 2; and GK1.5, anti-CD3T4; all from American Type Culture Collection, Manassas, VA) and rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) on day 0. These cells were cultured overnight in CM to remove the adherent macrophages, and then nonadherent cells were placed in fresh CM containing rm granulocyte macrophage-colony stimulating factor (GM-CSF; 1000 U/ml) and rmIL-4 (1000 U/ml; Shering-Plough, Kenilworth, NJ; DC media) on day 1. Cells were generally harvested on day 6. BM-DCs were defined by morphology, phenotype, and strong mixed lymphocyte reaction-stimulating activity. Phenotypic analysis by flow cytometry showed a high expression of CD11b, CD11c, CD80, CD86, as well as major histocompatibility complex class I and class II in the majority of the cultured cells (60–95%) [19]. These DCs were used for the subsequent experiments as immature DCs.

Flow cytometry

For phenotypic analysis of DCs, phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal Ab (mAb) against murine and human cell-surface molecules [CD11b, CD11c, CD80, CD86, Gr-1, H-2K^b, I-A^b for murine, CD1a, CD14, CD40, CD54, CD80, CD86, human leukocyte antigen (HLA)-ABC, HLA-DR, and appropriate isotype controls, all from PharMingen, San Diego, CA] were used. DCs were stained and analyzed on a FACScan using LYSIS II software (Becton Dickinson, Mountain View, CA) as described previously [19].

Purification of human CD34⁺ progenitor cells from BM

Human BM cells were harvested after informed consent. Mononuclear cells (MNC) were purified from human BM cells using density centrifugation with lymphocyte separation medium (LSM; Litton Bionetics, Kensington, MD). These cells were applied for immunoaffinity column (CellPro, Inc., Bothell, WA) after lysis of erythrocytes to purify CD34⁺ cells [20]. Purity of CD34⁺ cells was more than 85%, as determined by flow cytometry. These CD34⁺ cells were frozen down in liquid nitrogen until used for DC generation.

Generation of human CD34⁺ progenitor cell-derived DCs

On day 0, CD34⁺ cells (5×10^3 cells) were thawed and cultured in six-well plates with cRPMI 1640 with GM-CSF (500 U/ml), tumor necrosis factor α (50 ng/ml), stem cell factor (R&D Systems, Minneapolis, MN; 10 ng/ml), and follicular lymphoma (Immunex, Seattle, WA; 50 ng/ml) as described previously [21, 22]. Half of the medium was changed, and the cells were split at 1:2 every 3–4 days. On day 14, floating and loosely adherent cells were harvested and used as immature DCs, which were defined by morphology, phenotype, and strong mixed lymphocyte reaction-stimulating activity. These cells were highly positive for classes I and II, CD1a, CD40, CD80, and CD86 (39–91%) and weakly positive for CD83 (15–25%) [23].

Generation of monocyte-derived DCs

Leukocyte concentrates from healthy donors were harvested after informed consent and separated into peripheral blood MNC (PBMC) by density gradient centrifugation in LSM. These cells were suspended at 1×10^6 cells/ml and cultured in six-well plates for 1 h. After being washed twice, the adherent cells were cultured with GM-CSF (500 U/ml) and IL-4 (250 U/ml) for 7 days to generate monocyte-derived DCs [24–26].

MLR in mice and humans

Splenocytes were harvested from BALB/c mice (H-2^b). T cells were purified by T cell enrichment column (R&D Systems) after lysis of contaminating erythrocytes. The purity of T cells was $\geq 90\%$. T cells (2×10^5 cells) were cultured in 96-well plates with various numbers of BM-DCs irradiated with 1500 rad from C57BL/6 mice (H-2^b). On day 3, 50 μ l culture supernatants were collected for measurement of NO and enzyme-linked immunosorbent assay (ELISA) of IFN- γ . ³H-Thymidine (TdR; 1 μ Ci; ICN Radiochemicals, Costa Mesa, CA) was pulsed for the final 18 h, the culture was terminated, and the incorporation of ³H-TdR was measured as previously reported [27].

Human PBMC were purified by density centrifugation with LSM. T cells were purified by a T cell enrichment column (R&D Systems) after lysis of contaminating erythrocytes (purity $\geq 90\%$) [25]. These T cells (2×10^5 cells) were cultured in 96-well plates with various numbers of allogeneic CD34⁺ cell-derived DCs irradiated with 1500 rad for 5 days. ³H-TdR incorporation of the reactive T cells was measured as mouse MLR.

Measurement of NO

Human and mouse DCs were stimulated with IFN- γ (1000 U/ml) and/or LPS (1 μ g/ml) for 48 h. Their supernatants were harvested, and NO₂ production was assessed using the Griess method [28, 29]. Briefly, aliquots of culture supernatant (50 μ l) were incubated with 50 μ l Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H₂PO₄) at room temperature (RT) for 10 min. The absorbance was measured at 550 nm in an automated plate reader. The concentration was determined with reference to a standard curve of sodium nitrite.

ELISA for mIFN- γ

The concentration of IFN- γ was measured by ELISA (PharMingen) [30]. The lower limit of detection was 15.6 pg/ml. Briefly, microtiter plates were coated overnight with anti-mouse or human IFN- γ mAb at 4°C. The plates were then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and blocked with PBS containing 10% FBS at RT for 2 h. After washing, the standards and samples were added in the wells and incubated at RT for 4 h. The wells were then washed and incubated with the biotinylated second Ab at

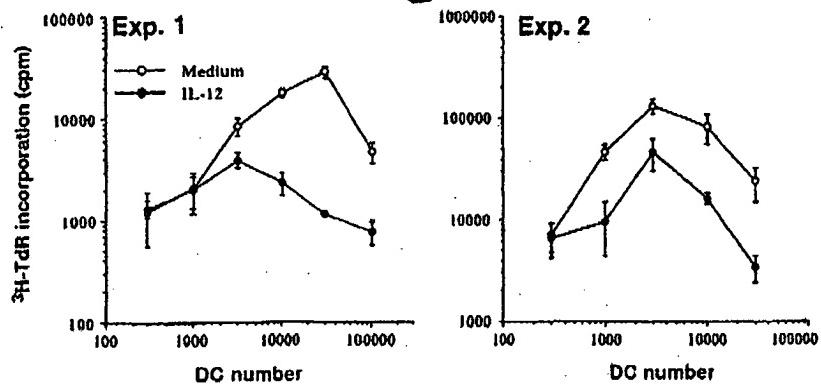


Fig. 1. The suppressive effect of IL-12 on allogeneic MLR in mice. T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated (1500 rad) DCs for 4 days. The rIL-12 (1 ng/ml) was added at the initiation of culture. ^{3}H -TdR was pulsed for the final 18 h. Data are presented as mean \pm SD of triplicate culture.

RT for 45 min. Finally, the avidine-peroxidase and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) substrate were added in the well, and the absorbance at 490 nm was determined with an ELISA plate reader (Titertek Multiscan, Meckenheim, Germany).

Generation of alloreactive CTL

Mouse alloreactive CTLs were generated as described previously [31]. Briefly, splenocytes were harvested, and T cells were purified as described above. T cells (2×10^6 cells) from BALB/c mice ($H-2^d$) were added in 24-well plates to irradiated (1500 rad) DCs (6.7×10^4 cells) from C57BL/6 mice ($H-2^b$; T cell:DC ratio = 30). After 5 days culture, T cells were harvested, and their lytic activity was assessed using a standard 4 h- ^{51}Cr release assay against EL-4 ($H-2^d$). The cytotoxicity of these alloreactive CTLs against P815 ($H-2^k$) was reproducibly less than 5%.

Human alloreactive CTLs were generated from peripheral blood lymphocyte-derived T cells. T cells (2×10^6 cells) purified as described above were cultured with allogeneic irradiated (1500 rad) DCs (6.7×10^4 cells) generated from CD34 $^{+}$ cells for 5 days. The cytotoxicity of these stimulated T cells was assessed for the same allogeneic DCs used as stimulators. These stimulated T cells did not show any cytotoxicity against autologous DCs generated from PBL with GM-CSF and IL-4.

The cytotoxicity of alloreactive CTL

The cytotoxicity was determined using a standard 4 h- ^{51}Cr release assay as described previously [26, 27]. In brief, 10^6 of each of the target cells was labeled with $100 \mu\text{Ci} \text{Na}_2^{51}\text{CrO}_4$ for 1 h. After washing twice, these effector and target cells were plated at an appropriate effector:target ratio in 96-well, round-bottom plates. The supernatant (100 μl) was collected after 4 h of incubation, and the radioactivity was counted with a γ -counter. The percentage of the specific lysis was calculated using the following formula: % specific lysis = $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$.

Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student's *t*-test. The difference was considered significant when the *P* value was less than 0.05.

RESULTS

Exogenous IL-12 suppressed the primary allogeneic MLR mediated by DCs in mice but not in humans

First, we tested the effect of IL-12 on the allogeneic MLR in mice and humans. There are no detectable IL-12 p70 heterodimers in mouse and human MLR in our system (data not shown), indicating that IL-12 is not mainly involved in alloreactive CTL generation in mouse MLR as described previously [32, 33]. It is interesting that the addition of exogenous IL-12 (1 ng/ml) suppressed MLR mediated by DCs in mice (Fig. 1). The dose dependence of IL-12-mediated suppression of MLR was confirmed in Figure 2A. IL-12 of 1 pg/ml or more was effective in inhibiting MLR. These suppressive effects were clearly found when a high number of DCs (≥ 3000 cells/well) were used for MLR as a stimulator and when IL-12 was added at the initiation of MLR (data not shown). However, when human DCs and T cells were used for MLR, IL-12 rather enhanced alloreactive T cell responses, consistent with previous reports [34] (Fig. 2B). As it has been clearly demonstrated that IL-12 is one of the growth factors of T cells [1, 2], these suppressive effects of mouse MLR by IL-12 seem to be a result of the indirect effect of IL-12 through other factors induced in MLR. IL-12 is known to stimulate IFN- γ production in T and NK cells [1, 2]. Therefore, we examined the production of IFN- γ in MLR as parallel experiments. As compared with the different effects of IL-12 on alloreactive T cell proliferation in mice and humans, IL-12 equally stimulated IFN- γ production in mouse and human MLR (Fig. 3), although the high number of DCs mediated the slight suppression of IFN- γ production in mice. These results suggested that the second factor induced

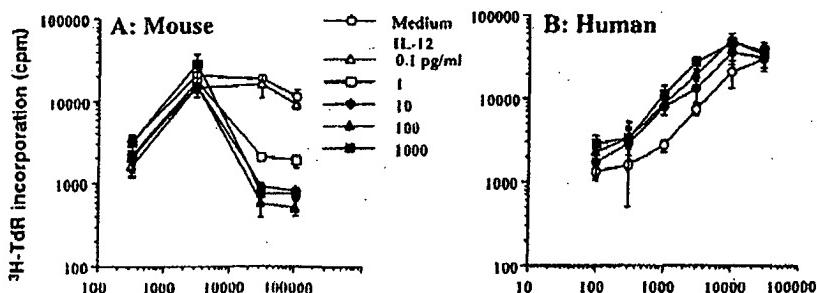


Fig. 2. Addition of IL-12 inhibits the generation of allogeneic MLR in mice but not in humans. Murine (A) and human (B) T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated (1500 rad) DCs for 4 days. The various doses of IL-12 were added at the initiation of culture. ^{3}H -TdR was pulsed for the final 18 h. Data in MLR are presented as mean \pm SD of triplicate culture. Similar results were obtained in three separate experiments.

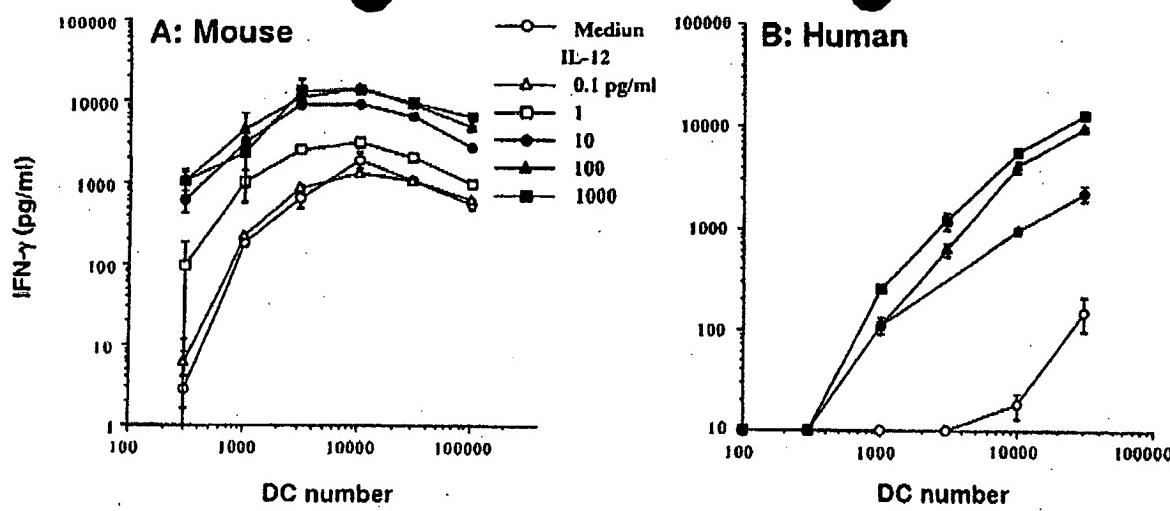


Fig. 3. IFN- γ production in murine and human MLR stimulated by DCs and IL-12. Murine (A) and human (B) T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated (1500 rad) DCs for 4 days. The various doses of IL-12 were added at the initiation of culture. The supernatant (50 μ l) was harvested before the addition of ^{3}H -TdR and assessed for IFN- γ production with ELISA as described in Materials and Methods. Data are presented as mean \pm SD of triplicate culture. Similar results were obtained in three separate experiments.

by IFN- γ might be responsible for the suppressive effects of IL-12 on mouse MLR.

Addition of IFN- γ suppressed the allogeneic MLR by DCs in mice, and L-NMMA completely reversed the suppressive effects of IFN- γ

To clarify the mechanisms involved in IL-12-mediated suppression of MLR in mice, we next tested the effect of an addition of IFN- γ on MLR by DCs in mice. It is interesting that the addition of IFN- γ also suppressed the allogeneic MLR in mice (Fig. 4A). These effects were reproducibly observed when more than 10 U/ml IFN- γ was used. A recent report by Bonham et al. [15] showed that murine DCs, irrespective of their maturation stage, could produce NO in response to IFN- γ and/or LPS as well as in the allogeneic MLR. In this report, NO was shown to be the mediator for DC apoptosis. However, previous reports refer to the important role of NO as the

inhibitor of T cell proliferation [35–38]. To examine whether NO played an important role in IFN- γ -mediated suppression of mouse MLR, we tested the effect of an addition of L-NMMA, an inhibitor of NO synthase (NOS). As shown in Figure 4B, L-NMMA (0.5 mM) completely reversed the suppressive effect of IFN- γ on MLR.

L-NMMA completely abrogated the suppressive effects of IL-12 on murine allogeneic MLR

Next, the effect of L-NMMA on the IL-12-mediated suppression of mouse MLR was examined. An addition of L-NMMA enhanced allogeneic MLR stimulated with a high number of DCs in mice, as previously reported [15] (Fig. 5). Furthermore, the addition of L-NMMA completely abrogated the IL-12-mediated suppression of mouse MLR (Fig. 5). IFN- γ production, which was induced by the addition of IL-12, was further enhanced by L-NMMA in mouse MLR. In contrast, L-NMMA

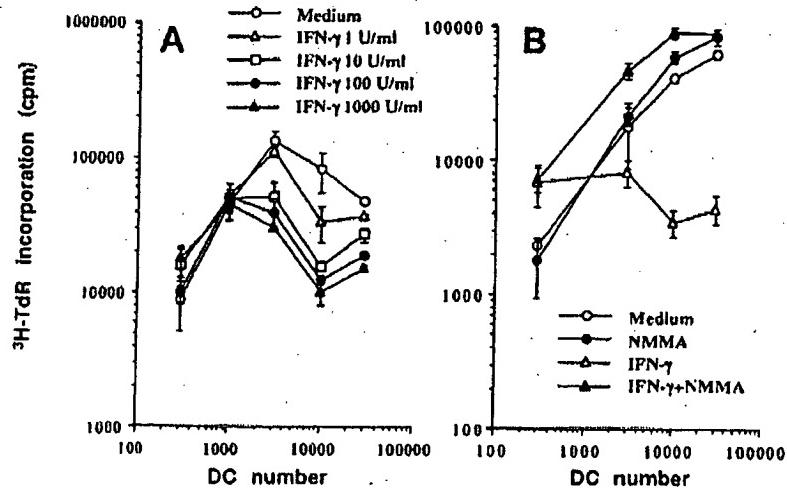


Fig. 4. Addition of IFN- γ inhibits the induction of allogeneic MLR in mice, and L-NMMA abrogates the IFN- γ -mediated suppression of MLR. T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated DCs for 4 days. The various doses of IFN- γ were added at the initiation of culture (A). L-NMMA (0.5 mM) was also added in MLR in the presence of 1000 U/ml IFN- γ (B). Data in MLR are presented as mean \pm SD of triplicate culture. Similar results were obtained in two separate experiments.

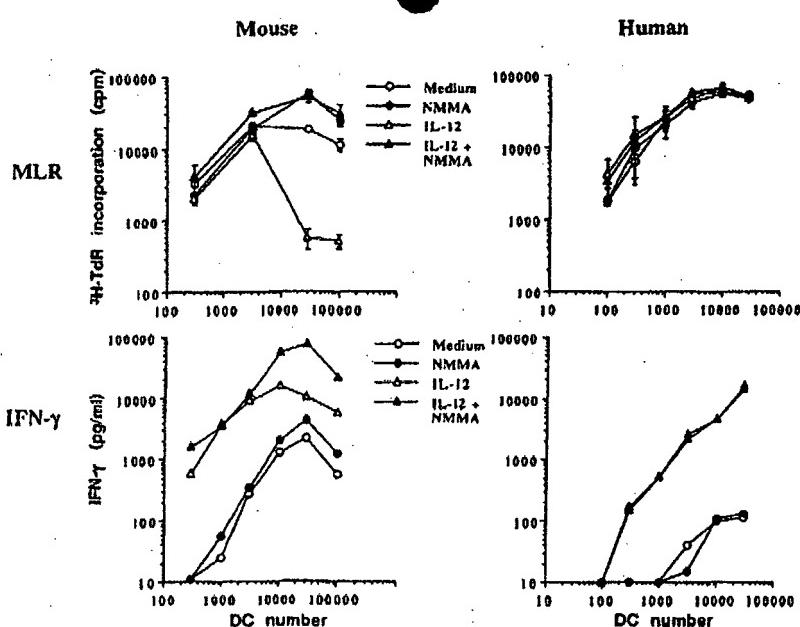


Fig. 5. L-NMMA abrogates the IL-12-mediated suppression of mouse allogeneic MLR, but human allogeneic MLR was not affected by the addition of L-NMMA. T cells (2×10^5 cells/well) were cultured with the indicated number of irradiated DCs for 4 days. L-NMMA (0.5 mM) was added to the allogeneic MLR of mice and humans. IL-12 (1 ng/ml) was used in these experiments. IFN- γ production in MLR was also assessed with ELISA. Data in MLR are presented as mean \pm SD of triplicate culture. Similar results were obtained in three separate experiments.

did not produce any effect on the proliferation and IFN- γ production in human MLR (Fig. 5).

Murine BM-DCs but not human DCs can produce a significant amount of NO in response to various stimuli

The data presented above, together with previous reports, suggested that murine DCs produce NO in MLR, which inhibits the growth of alloreactive T cells. We compared the production of NO by murine and human DCs. Figure 6 shows that a significant level of NO is detectable in mouse MLR but not in human MLR, and the addition of L-NMMA completely

suppressed NO production in mouse MLR. Furthermore, IL-12 markedly enhanced NO production in mouse MLR, whereas no detectable level of NO was found, even in the existence of exogenous IL-12 in human MLR. Next, we tested which cell population can produce NO in MLR. Table 1 shows that murine BM-DCs produce a significant amount of NO in response to IFN- γ and/or LPS, but human CD34 $^{+}$ cell- and monocyte-derived DCs do not. In this experiment, T cells from neither mice nor humans produced a detectable level of NO (data not shown). These results suggest that NO produced by DCs might play an important role in the immune response in the mouse system, especially in the presence of IL-12.

Effect of SNAP on murine and human MLR

To rule out the possibility that human T cells were insensitive for NO, we examined the effect of addition of NO in human

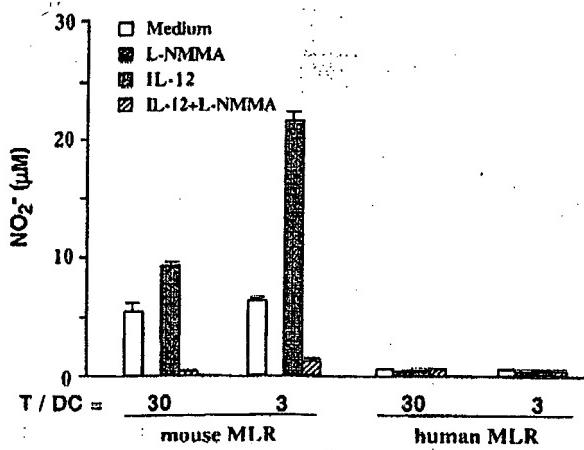


Fig. 6. NO $_2^-$ was detected in mouse MLR with DCs but not human MLR, and the addition of IL-12 significantly enhanced NO production in mouse MLR. T cells (2×10^5 cells/well) were cultured with the irradiated (1500 rad) DCs at the indicated T cell:DC ratio (T/DC) for 4 days. Their supernatant was harvested to measure the NO production in MLR using the Griess method as described in Materials and Methods. Similar results were obtained in two separate experiments.

TABLE 1. Murine BM-DCs but Not Human DCs Produce NO in Response to Various Stimuli*

Treatment	NO $_2^-$ (μM)		
	Murine BM-DC	CD34 $^{+}$ -DC	mo-DC
Exp. 1			
Medium	<1.0	<1.0	<1.0
IFN- γ	1.33 \pm 0.16 ^b	<1.0	<1.0
LPS	1.87 \pm 0.07	<1.0	<1.0
LPS + IFN- γ	26.03 \pm 0.76	<1.0	<1.0
Exp. 2			
Medium	<1.0	<1.0	<1.0
IFN- γ	6.19 \pm 1.71	<1.0	<1.0
LPS	24.22 \pm 1.69	<1.0	<1.0
LPS + IFN- γ	86.85 \pm 2.31	<1.0	<1.0

* Murine and human DCs (1×10^5 cells/well) were stimulated with IFN- γ (1000 U/ml) and/or LPS (10 μ g/ml) for 48 h. The supernatant was harvested and used for measuring NO $_2^-$ with Griess reaction (sensitivity > 1.0 μ M).
^b Data are mean \pm SD of triplicate samples. mo, Monocyte.

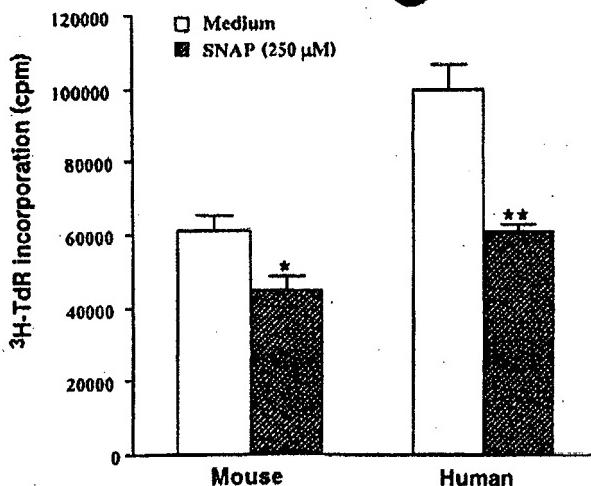


Fig. 7. Effect of addition of SNAP (NO donor) on murine and human MLR. T cells (2×10^5 cells/well) were cultured with irradiated (1500 rad) DCs (1×10^4 cells/well) for 4 days. SNAP (250 μ M) was added at the initiation of culture. ^{3}H -TdR was pulsed for the final 18 h. Data are presented as mean \pm SD of triplicate culture. *, $P < 0.01$ as compared with the group without SNAP; **, $P < 0.001$ as compared with the group without SNAP.

MLR using SNAP as a NO donor [15]. The 250 μ M SNAP was added in murine and human MLR stimulated by DCs at the initiation of culture. As shown in Figure 7, the proliferation of murine and human T cells was significantly inhibited by the treatment with SNAP, indicating that murine and human T cells were NO-sensitive.

IL-12 enhanced alloreactive CTL activity in human MLR, but L-NMMA is required to generate mouse allogeneic CTL in the presence of IL-12 in vitro

Finally, we tested the effect of IL-12 with or without L-NMMA on alloreactive CTL induction in mice and humans in vitro. As described in Materials and Methods, mouse allospecific CTL

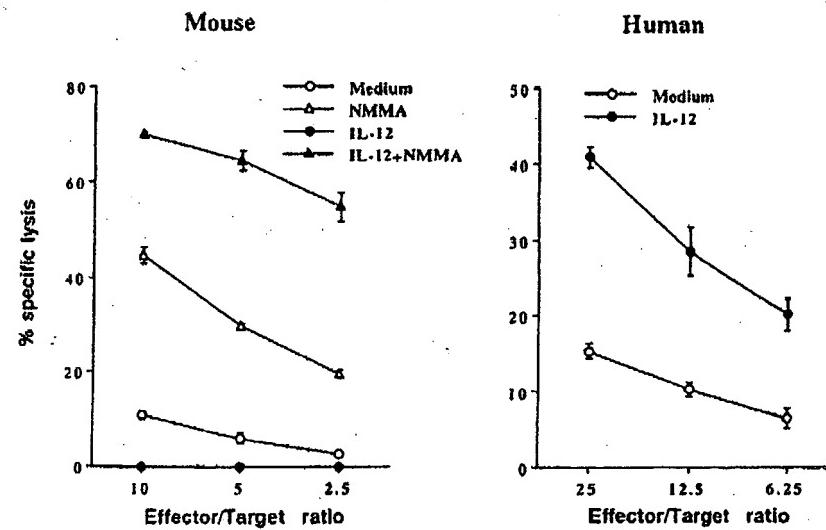
activity was determined against EL-4 (H-2^b) as compared with the control syngeneic target P815 (H-2^d). Human alloreactive CTL activity was examined against allogeneic DCs used in MLR as compared with control, autologous DCs. As previously reported [34], IL-12 (1 ng/ml) significantly enhanced alloreactive CTL activity in human MLR, whereas we could not detect any enhancing effects of IL-12 on the generation of murine alloreactive CTL in vitro (Fig. 8); rather, IL-12 strongly inhibited the allospecific CTL generation. It is interesting that the addition of L-NMMA to murine allogeneic MLR without IL-12 enhanced allospecific CTL activity. Furthermore, L-NMMA addition to murine MLR with IL-12 clearly overcame IL-12-mediated suppression of murine alloreactive CTL induction, resulting in the generation of the highest alloreactive CTL activity among all the groups tested. The CTL activity induced by the treatment of L-NMMA + IL-12 was significantly higher than that of medium + L-NMMA. In this experiment, cytotoxicity against P815 was less than 5%, indicating strict allospecificity of these CTL (data not shown).

DISCUSSION

In this report, we examined the effect of IL-12 on the primary, allogeneic MLR mediated by DCs in mice and humans. It is interesting that the addition of a high dose of rmIL-12 suppressed allogeneic MLR and CTL induction in mice but enhanced them in humans. An analysis of the mechanisms involved in the suppressive effect of IL-12 showed that NO played a critical role as an inhibitory factor for alloreactive T cell response by DCs only in mouse MLR.

IL-12 is known to play an important role in promoting Th1 differentiation and cellular immune responses. The numerous studies demonstrated the antitumor effects of IL-12, whereas the detail mechanisms remained unclear as a result of the complex effects of IL-12. The recent report by Andrews et al. [39] demonstrated that the antitumor effects of adenovirus expressing IL-12 (AdV-mIL-12) were mainly mediated by the

Fig. 8. IL-12 enhanced alloreactive CTL activity in human, but L-NMMA was required for the enhancement of alloreactive CTL in mouse MLR with IL-12. Mouse T cells (2×10^6 cells) from BALB/cj mice (H-2^b) were added in 24-well plates to irradiated (1500 rad) DCs (6.7×10^4 cells) from C57BL/6 mice (H-2^b; T cell:DC ratio=30) in the presence or absence of IL-12 (1 ng/ml) with or without L-NMMA (0.5 mM). After 5 days culture, T cells were harvested for a 4 h ^{51}Cr release assay against EL-4 (H-2^b). Human alloreactive CTL were generated from PBL-derived T cells. T cells (2×10^6 cells) were cultured with allogeneic irradiated (1500 rad) DCs (6.7×10^4 cells) generated from CD34⁺ progenitor cells in the presence or absence of IL-12 (1 ng/ml) for 5 days. The cytotoxicity of these stimulated T cells was assessed for the same allogeneic DCs as the stimulators. Data are presented as mean \pm SD of triplicate culture. Similar results were obtained in two separate experiments.



inhibition of angiogenesis, as intratumoral AdVmIL-12 treatment effectively regressed the established tumor even in immune-deficient mice, including CD4- or CD8-depleted, severe combined immunodeficiency, Beige, and RAG-2-, NKT-, CD28-, and perforin-knockout mice. Furthermore, Boggio et al. [40] also reported that IL-12 prevented the spontaneous tumor development through antiangiogenic effects. Conversely, Horvath-Arcidiacono et al. [41] reported the discrepancy between the inhibitory effects of tumor growth and CTL generation in IL-12-treated mice. They found that administration of IL-12 in tumor-bearing mice eventually resulted in the rejection of tumors, but CTL activity was reduced despite the enhancement of serum IFN- γ . Noguchi et al. [9] also showed that administration of a low dose of IL-12 (1 ng/mouse) enhanced CTL activity specific for the mutated p53 peptide (234 CM) and regressed the MethA tumor expressing it, whereas a high dose of IL-12 (100 ng/mouse) suppressed 234CM-specific CTL generation. Orange et al. [10] also reported that the administration of IL-12 decreased antiviral CTL activity in the LCMV infection model. Furthermore, the report presented by Piccotti et al. [11] demonstrated that IL-12 antagonism using anti-IL-12 Ab or IL-12 p40 homodimer exacerbated cardiac allograft rejection in mice. Koblish et al. [13] recently elucidated the mechanisms involved in IL-12-mediated suppression on cellular immune responses. They reported that NO produced by adherent cells in spleen was an important factor in the IL-12-mediated immune suppression, as a NOS inhibitor completely abrogated it [13]; whereas the concentration of NO was not measured. In this study, we confirmed and extended their study using allogeneic MLR stimulated by DCs and demonstrated that murine DCs as well as macrophages were potential producers of NO. Bonham et al. [15] and Lu et al. [42] also demonstrated NO production of murine BM-DCs and that L-NMMA enhanced allogeneic MLR when a high number of DCs were used [15, 42].

These results presented above support the possibility that IL-12 has the suppressive effects on allogeneic CTL generation by enhancing NO production. It is not surprising that NO suppresses the mitogenic activity of T cells and development of allospecific CTLs, as the previous reports showed evidence that NO produced by macrophages was a suppressive factor of the proliferation of lymphocytes [43–45]. However, Piccotti et al. [46] verified the enhancement of allogeneic MLR in the presence of IL-12. Bloom and Horvath [47] reported that IL-12 did not affect allogeneic MLR. These discrepancies between the previous reports and ours might be a result of the differences of stimulator used in MLR. BM-DCs were used in our experiments and splenocytes in the other as a stimulator. Splenocytes contain a DC population, but the percentage of DCs in the spleen is 1.0–1.6% [48]. DCs are stronger stimulators in MLR when compared with splenocytes. Therefore, the stimulation by DCs might lead to a higher production of NO and the subsequent suppression of MLR. As DCs but not other APCs are known to play a key role in the induction of primary immune responses [14], experiments with DCs seem to be more important to reflect the host immune response. Our data clearly demonstrated that NO is a suppressive factor presumably produced by mouse DCs in MLR. In addition to the data of NO₂⁻ production, the blocking experiments with L-NMMA corroborated the inhibitory effect of NO on mouse MLR. Furthermore, the treatment with L-NMMA clearly enhanced allogeneic CTL activity. These data suggest that the combined use of IL-12 and L-NMMA might be effective for treatment of tumors in mice. In fact, Koblish et al. [13] reported that inhibition of NOS function using L-NMMA enhanced IL-12-induced delay of SCK tumorigenesis. Furthermore, as Gajewski and co-workers [49] reported, the use of a low dose of IL-12 might be better in combination with DC-based immunotherapy.

In the present study, exogenous IFN- γ also suppressed allogeneic MLR, and the addition of L-NMMA abrogated IFN- γ -mediated suppression. There are many controversial reports in which IFN- γ enhances or inhibits CTL generation. The recent study using mice with the IFN- γ gene knocked out showed that allospecific CTL activity of IFN- γ ^{-/-} mice was higher than that of IFN- γ ^{+/+} mice, indicating that the existence of IFN- γ somehow inhibits the generation of allospecific CTL [50]. As IFN- γ stimulates NO production, it is likely that these results might have been affected by the condition of NO production in culture.

Conversely, exogenous human IL-12 has been reproducibly reported to enhance antigen-specific human CTL induction by DCs from CD8⁺ T cells in vitro [34, 39]. Chouaib et al. [34] showed that IL-12 was involved in human allogeneic MLR and the addition of rhIL-12-enhanced, allospecific CTL activity using a blocking experiment with anti-human IL-12 antibody. Consistent with these results, we demonstrated in this report that IL-12 (1 ng/ml) enhanced allospecific CTL activity in humans. This difference between murine and human CTL induction by IL-12 was a result of the different production of NO by DCs. The present study was the first report describing that human CD34⁺ cell- and monocyte-derived DCs did not produce a significant amount of NO in response to LPS and/or IFN- γ as well as in allogeneic MLR.

These results suggest that NO production by APCs including macrophages and DCs is an important factor regulating the effect of IL-12 in mice. The combined use of L-NMMA might reverse the suppressive effects of IL-12 on the induction of CTLs in mice and more closely mimic immune responses mediated by IL-12 in humans. As NO induced by IL-12 in vivo is likely to be less in humans, the antitumor effect mediated by IL-12 via cellular immune responses might be expected in humans rather than in mice.

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Zinc Inhibits the Mixed Lymphocyte Culture

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ABSTRACT

The mixed lymphocyte culture (MLC) is an established clinical method for bone marrow transplantation, as it serves as an *in vitro* model for allogenic reaction and transplantation. We previously showed that cytokine release into the supernatant is a more specific and sensitive parameter for cross-reactivity in the MLC than the common measurement of cell proliferation. Therefore we tried to find an inhibitor of the MLC *in vitro* with the least side effects *in vivo*, measuring interferon (IFN)- γ as one of the most important cytokines in posttransplant medicine. Earlier studies showed that zinc is an important trace element for immune function with both stimulatory and inhibitory effects on immune cells. We found that slightly elevated zinc concentrations (three to four times the physiological level), which do not decrease T-cell proliferation *in vitro* nor produce immunosuppressive effects *in vivo*, suppress alloreactivity in the mixed lymphocyte culture. In this report we analyzed the mechanism whereby zinc influences the MLC to possibly find a nontoxic way of immunosuppression.

Index Entries: Mixed lymphocyte culture (MLC); mixed lymphocyte reaction (MLR); trace elements; zinc.

INTRODUCTION

The mixed lymphocyte culture (MLC) is a well-established and important tool for determination of compatibility between host and donor

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in transplantation medicine, as it serves as an in vitro model for allogenic reaction (1,2). It is common to measure T-cell proliferation in the MLC, but it was recently shown that cytokines are more specific and sensitive parameters for the prediction of a possible graft rejection, as they play a critical role in the posttransplant response (3-5). The TH1-cytokine interferon- γ (IFN- γ) was identified as the most important factor within the cytokine cascade in the MLC (6). It is known to induce cytotoxic T-lymphocytes (CTL) (7) by enhancing the expression of both major histocompatibility complex (MHC) class I and MHC class II molecules (8). The IFN- γ response mainly depends on HLA-DR differences and it therefore well represents reactivity between two individuals in the MLC (9).

In transplantation medicine, cyclosporin A, FK506, and other substances are used to prevent graft rejection. In vitro experiments revealed an inhibition of the MLC (10), but, unfortunately, all of these immunosuppressants show a wide range of toxicities in vivo, such as nephrotoxicity, neurotoxicity, and, probably, carcinogenicity (11-13). As we are beginning to understand the molecular mechanisms of cyclosporin A and FK506 function better and better, one of the major aims is to find similar substances with less toxicity.

Zinc within the physiological range (12-16 μ M) is an important trace element for immune function (14). Zinc deficiency in vivo could be linked to various clinical symptoms such as impaired immune response with regard to decrease in number, differentiation, and function of T-lymphocytes and natural killer (NK) cells as well as decreased activation of monocytes and phagocytosis by macrophages, resulting in a high incidence of bacterial, viral and fungal infections. These symptoms, in the most severe form shown in the hereditary disease acrodermatitis enteropathica caused by malabsorption of zinc, are completely reversible after adequate substitution of zinc (15). On the other hand, high concentrations of zinc (about eight times the physiological level) led to cytotoxic effects with impairment of all T-cell functions, and inhibition of monokine induction by superantigens such as zinc is also important for the binding of some bacterial superantigens to the β -chain of the MHC class II molecule (16,17). Optimal immune-cell function hence requires a well-balanced zinc level.

In the following study, we investigated whether zinc is able to impair alloreactivity in the MLC at concentrations with neither cytotoxic effects in vitro nor toxic side effects in vivo.

MATERIALS AND METHODS

Preparation of Lymphocyte Cultures

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by density centrifugation over Ficoll-Hypaque (Biochrom, Berlin, Germany), washed twice with phosphate-buffered saline (PBS, Gibco, Berlin, Germany) and resuspended in RPMI-1640 medium (Biochrom) supplemented with 10% heat-inactivated fetal calf

serum (FCS, low endotoxin, myoclone quality; Life Technologies, Eggenstein, Germany), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all obtained from Biochrom, Berlin, Germany). The cells were adjusted to a final concentration of 2×10^6 cells/mL. Equal volumes of cell suspensions of two donors were seeded in samples to a final volume of 1 mL into pyrogen-free 24-well culture plates (Falcon, Heidelberg, Germany). For controls, 1 mL of the adjusted cell suspension was cultured separately. The cultures were incubated for 5 d at 37°C in a 5% humidified CO₂ atmosphere after addition of the appropriate amount of zinc.

Zinc Preparations

Zinc sulfate (Sigma, Deisenhofen, Germany) was dissolved in sterile water to achieve a zinc stock solution of 10 mM. This solution was further diluted in unsupplemented protein-free medium (PFM, Ultradoma, BioWhittaker) at a ratio of 1 to 2 and then sterile filtered. To achieve the final concentrations, PFM was used. The zinc solution was added to the cultures in a volume of 10% of the final culture volume.

Determination of Cytokines

The culture supernatants were harvested after 5 d and stored at -80°C. The quantification of the cytokine release into the supernatant was performed by enzyme-linked immunosorbent assay (ELISA) technique (for IFN-γ provided by Bender Med Systems, Vienna, Austria). Results were measured in picograms per milliliter at 450 nm using an ELISA plate reader (Anthos Labtec, Salzburg, Austria).

Flow Cytometry

Propidium iodide (PI) staining was performed by using a stock solution of 1 mg/mL (PI, Sigma). Cells (1×10^6 /mL) were incubated with 10 µL of PI stock solution for 20–30 min to allow intercalation of PI in double-stranded DNA. Finally, PI staining was measured at a wavelength of 620 nm in a flow cytometer (Coulter, Krefeld, Germany).

Statistical Analysis

The results are expressed as median values. The significance is taken by Student's *t*-test analysis.

RESULTS

Influence of Zinc on Mixed Lymphocyte Cultures

We harvested the supernatants of zinc-supplemented mixed lymphocyte cultures (MLC) on d 5, proven to be the maximum of the IFN-γ secretion (18). Analyzing IFN-γ release in 20 MLC experiments supplemented with different concentrations of zinc, we found expected amounts of IFN-

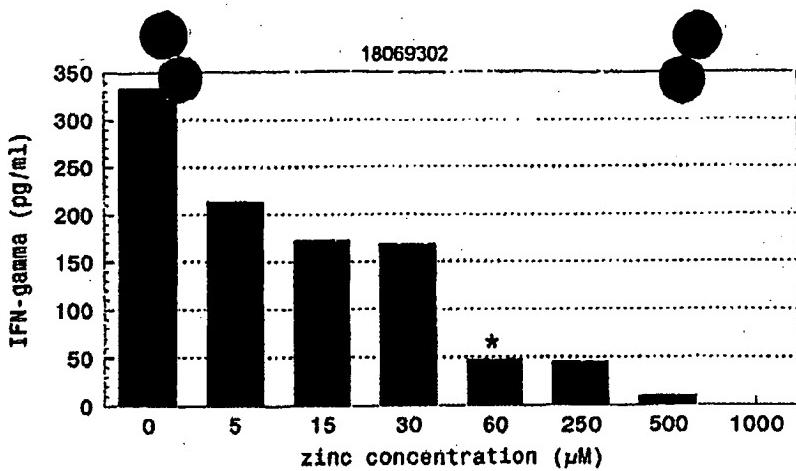


Fig. 1. IFN- γ secretion in the MLC after zinc supplementation. Zinc concentrations up to 1 mM were added to mixed lymphocyte cultures; controls remained unsupplemented. Secretion of IFN- γ in the culture supernatants was determined by ELISA after 5 d of culture. Median values of $n = 20$ experiments are expressed in picograms per milliliter. Significance was calculated by the Student's *t*-test (* $p = 0.0017$).

γ (334 pg/mL) in the supernatant of control MLC without zinc addition, whereas increasing zinc concentrations led to a dose-dependent reduction of the IFN- γ level. At 60 μM , the IFN- γ production was significantly diminished (48 pg/mL, $p = 0.0017$); at 500 μM , no IFN- γ was detectable (Fig. 1).

In order to prove our hypothesis that this result—that zinc concentrations of 60 μM inhibit the MLC—was the result of a specific effect in the MLC and not to a loss of T-cell vitality, we added zinc concentrations of up to 5 mM to PBMC and measured cell viability by flow cytometry after an incubation time of 48 h. Figure 2 shows that 93.2% of the cells are still vital after addition of 50 μM zinc and 92.3% with medium supplementation of 100 μM zinc compared to controls without zinc addition with 91.3% viability. Zinc concentrations as high as 250 μM causes a reduction of cell survival of 33% (Fig. 2).

For further analysis of possible mechanisms responsible for this inhibition, we preincubated PBMC with 50 μM zinc for 20 min and then cocultured these two populations in the MLC. The results reveal a marked influence of the point of time at which zinc is added to the culture: Preincubation of PBMC led to a greater reduction of IFN- γ than simultaneous zinc supplementation to the MLC (Fig. 3).

DISCUSSION

The human mixed lymphocyte culture (MLC) is an important method to test donor-recipient compatibility in bone marrow transplantation. It could be shown that cytokine release, especially IFN- γ , has a very good predictive value with regard to the transplantation outcome (3), as cytokines play a major role in the generation of an alloreactive immune response and

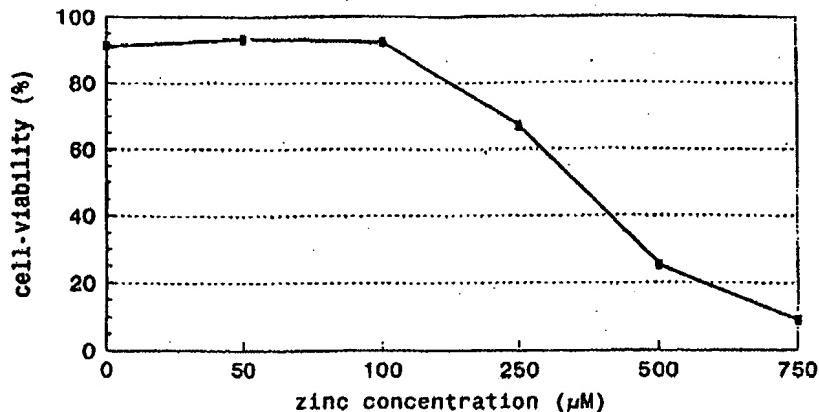


Fig. 2. Viability of PBMC after zinc supplementation. Zinc concentrations of up to 750 μM were added to unstimulated PBMC; controls remained unsupplemented. Cell viability was determined by flow cytometry after an incubation time of 48 h. One representative experiment is shown, values are expressed in percent of the total cell population.



Fig. 3. Effect of preincubation of PBMC with zinc. Zinc in a concentration of 50 μM was added to the MLC simultaneously (MLC + Zn); PBMC was preincubated with 50 μM zinc for 20 min and then cocultured in a MLC (PBMC + Zn); controls remained unsupplemented. Medians of $n = 10$ experiments are presented. Significance was calculated by the Student's *t*-test (* $p = 0.01$).

for the induction of graft rejection *in vivo* (4,5). Taking this *in vitro* model, it has always been the aim to inhibit proliferation of immune cells in order to find a way to prevent graft rejection in transplantation medicine. Landolfo et al. inhibited T-cell reactivity by the addition of anti-IFN- γ both *in vitro* and *in vivo* (19); others showed a reduced graft rejection rate by repeated injections of monoclonal anti-IFN- γ antibodies in a skin-, heart-, or pancreas-tissue transplantation situation (20-22).

In vivo substances like cyclosporin A or FK506 are broadly applied, as they are capable of prolonging graft survival. In vitro, they show an inhibitory effect on T-cell proliferation in the MLC (10). Yet, all of these therapeutical agents cause major side effects (e.g., nephrotoxicity, neurotoxicity, and others), which lead to a limitation of their use (11-13).

Zinc is an essential trace element with great influence on immune function. The physiological plasma level of zinc ranges from 12 to 16 μM . In our study, we applied zinc concentrations up to 100 μM , which can be reached by pharmacological application of zinc *in vivo* without causing side effects (23).

We found that zinc concentrations of 60 μM , four times the physiological level, inhibit alloreactivity in the MLC. It is unlikely that the reduction of IFN- γ is the result of a loss of T-cell activity, as it could be shown earlier that T-cells are still able to proliferate in medium supplemented with zinc concentrations as high as 100 μM (24). Furthermore, we analyzed the viability of the PBMC by flow cytometry, showing that a concentration of 250 μM is required to reduce cell viability by 33% (Fig. 2).

Increased zinc levels of over 100 μM cause unstimulated human PBMC to release cytokines (25). This stimulatory effect of zinc is only seen in the presence of accessory cells, especially monocytes, as mostly IL-1 proved to be an essential cosignal for T-cell activation by zinc. Higher concentrations of zinc impair all T-cell and monocyte function by inhibition of the IL-1 receptor type I-associated protein kinase (IRAK), thus blocking the intracellular signal transduction pathway at a very early stage (24).

In our study, we applied zinc in concentrations that neither show cytotoxic effects nor reach stimulatory level. Therefore, there seems to be a specific effect of zinc on the responding T-cells in the MLC.

The results of earlier studies proposed an oligoclonal pattern of T-cell stimulation in the MLC similar to T-cell activation by superantigens (3). Furthermore, a highly altered V β repertoire of T-cells infiltrating long-term rejected kidney allografts were described (26). Superantigens bind directly and partially with high affinity to major histocompatibility complex (MHC)-class II proteins, especially to HLA-DR. T-Cell activation is achieved by the formation of a complex of the V β -chain of the T-cell receptor (TCR), the MHC molecule, and the superantigen. This binding is regulated by zinc, as zinc itself does not interact with the MHC molecule directly (27). We previously showed that the HLA-DR and HLA-DQ-molecules have the greatest influence on cytokine release in the MLC and thus on the outcome of a transplantation *in vivo* (9).

There are two main possible explanations for the phenomenon described. First, zinc in the applied concentration could saturate the MHC and, therefore, prevent a binding between TCR and MHC. In order to prove this hypothesis, we preincubated PBMC with zinc and then cocultured these populations. If an extracellular mechanism were actually responsible for the inhibition of the MLC, we would expect no significant difference in IFN- γ secretion in either setting. Preincubation of PBMC

resulted in a markedly lower IFN- γ secretion than the culture of two PBMC populations with simultaneous zinc supplementation to the MLC (Fig. 3), so that it seems more likely that zinc interferes with the intracellular signal transduction in the MLC. Therefore, zinc may regulate the alloreactivity of T-cells and might be an explanation for increased preterm delivery and abortion in zinc-deficient pregnant women (28,29). As mentioned earlier, higher concentrations of zinc are able to block the intracellular signal transduction pathway by inhibition of IRAK. We propose that the stimulation of T-cells by an HLA-different cell population can be blocked by zinc via specific inhibition of phosphorylation processes, leading to a diminished signal transduction in the cell. This results, among other things, in reduced secretion of cytokines, which should lead to less graft rejection *in vivo*. Various protein kinases such as cAMP- and cGMP-dependent protein kinases as well as protein tyrosine kinases are involved in zinc-induced cell stimulation and zinc also influences gene expression of different immunologically relevant transcription factors such as nuclear factor (NF)- κ B and metallothionein transcription factor (MTF-1) as well as others. Which alteration of signal transduction zinc exactly inhibits the MLC remains the subject of further investigation. Because the MLC is inhibited by very low zinc concentrations, this inhibitory effect seems to be a specific pathway.

In conclusion, zinc could become an immunosuppressant in transplantation medicine without toxic side effects, which still leaves the immune system with the ability for phagocytosis. The infection rate will therefore be reduced compared to current immunosuppression. However, this has yet to be proven in *in vivo* transplantation models.

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